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Award Number: W81XWH-12-1-0176

TITLE: Promotion of Tumor-Initiating Cells in Primary and Recurrent Breast Tumors

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REPORT DATE: July 2013

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual	1 July 2012 - 30 June 2013

4. TITLE AND SUBTITLE **Promotion of Tumor-Initiating Cells in Primary and Recurrent Breast Tumors**

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0176

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Albert S. Baldwin, Ph.D.

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of North Carolina

Chapel Hill, NC 27599-7295

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Breast tumors, like other solid tumors, contain a subset of cells known as tumor initiating cells (TICs). TICs are important in tumor initiation and recurrence, metastasis, and are resistant to radiation and chemotherapy. Our data and work published by others indicate that the transcription factor NF-κB is likely to be important in the generation and maintenance of breast TICs. A major problem in breast cancer therapy is the issue of residual disease where therapy-resistant cancer cells remain dormant only to drive tumor recurrence years later. Here we propose to explore mechanisms whereby NF-κB is activated in breast cancer TICs and recurrent experimental tumors and the roles that IKK/NF-κB play in this process. Our hypothesis is that the IKK/NF-κB pathway is essential for the development and/or maintenance of breast cancer TICs, potentially through the promotion of EMT and the regulation of expression of genes which confer stemness. We hypothesize that inhibition of IKK/NF-κB will reduce or eliminate breast camcer TICs, blocking tumorigenesis. Furthermore, we hypothesize that the activation of NF-κB is an important component in the generation of recurrent diseases derived from residual disease. The Aims are to: (i) Explore the mechanistic roles for IKK/NF-κB in promoting basal-like and Her2+ tumor initiating cells. (ii) Test IKK inhibitors and mTOR inhibitors for effects on tumor growth and TIC phenotypes, (iii) Determine the requirement for the NF-κB, TGFβ and mTOR pathways in promoting the survival and recurrence of residual cancer cells.

15. SUBJECT TERMS
Breast cancer, tumor initiating cells, NF-kappaB, macrophages

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER *(include area code)*

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Attachments:

Manuscripts: Merkhofer et al (Oncogene 2010)
Kendellen et al (in review, Breast Cancer Research)
Abstract: DOD Era of Hope 2011

INTRODUCTION:

Tumor initiating cells (TICs) are proposed to drive the growth and metastatic properties of a tumor (Korkaya et al., 2011). One of the goals of this proposal is to determine if the IKK/NF-κB pathway is essential for the maintenance/expansion of basal-like and Her2+ TICs and, if so, to determine the signaling pathways that promote this activity. These breast tumor subtypes present distinct therapeutically difficult cancer (Brenton et al., 2005; Sorlie et al., 2003; Hu et al., 2006; Carey et al., 2006). Experiments utilize cell-based and animal tumor models. Additionally, we will determine the biological/signaling mechanisms that underlie IKK/NF-κB activity (Hayden and Ghosh, 2004; Karin, 2006; Basseres and Baldwin, 206) to promote the TIC phenotype. Previously others showed that NF-κB is activated in basal-like breast cancers (Yamaguchi et al., 2009). Currently we have shown that NF-κB is highly active in TICs isolated from two different basal-like cancer cell lines. Inhibition of NF-κB (through genetic and drug-based approaches) demonstrates that IKK/NF-κB is required for self-renewal of breast cancer TICs. Importantly, inhibition of NF-κB strongly blocked xenograft tumor formation, consistent with an inhibition of TIC function. Interestingly, we have found that both canonical and non-canonical NF-κB signaling contribute to breast cancer TIC maintenance/expansion. Finally, we showed that certain inflammatory cytokines whose genes are regulated by NF-κB (IL-6, and IL-1) promote the expansion of TICs. These findings have been published (Kendellen et al., *Oncogene* 2013). The completion of our proposed aims (Aims 1 and 2) may lead to new therapeutic options for basal-like breast cancer as well as a better understanding of causative mechanisms in oncogenesis and therapy resistance for this disease.

Residual breast cancer cells have the ability to survive following treatment and linger unrecognized in a presumed dormant state for up to 20 years before re-emerging as recurrent disease. Since recurrent breast cancer is typically fatal, the propensity of residual breast cancer cells to survive therapy and recur is one of the most important determinants of clinical outcome. We (Chodosh --- collaborator) have found that recurrent breast cancers that arise in genetically engineered mouse models frequently undergo EMT. Moreover, we have demonstrated that the EMT transcription factor, Snail, is markedly upregulated in recurrent mammary tumors, and that forced expression of Snail in primary tumors is sufficient to promote mammary tumor recurrence. We have also obtained evidence indicating that the NF-κB and TGFβ pathways are upregulated in recurrent mammary tumors. In aggregate, our findings suggest that the NF-κB and TGFβ pathways may contribute to the reservoir of residual cancer cells that give rise to recurrent breast cancers. The goals of Aim 3 of this proposal are to determine if the NF-κB, TGFβ and mTORC1 pathways are essential for the survival and recurrence of residual cancer cells from TICs.

Statement of Work/Aim 1 (Directed by Dr. Baldwin).

---Explore the involvement of NF-κB in promoting epithelial-mesenchymal transition as a regulatory mechanism in promoting TIC phenotype. Experimental approaches: inhibition of IKK/NF-κB in bulk cultures and CD44+ TICs, immunoblotting of breast cancer TICs for markers of EMT.

---Determine signaling pathways, important to breast cancer TICs, that promote the EMT phenotype and the upregulation of NF-κB. Experimental approaches: isolation of CD44+ cells, siRNA knockdown experiments, co-immunoprecipitation experiments related to TAK1/TAB/IKK interaction, immunoblotting for EMT markers.

---Characterize the NF-κB-dependent gene expression profile in breast cancer cell TICs. Determine if NF-κB promotes chemoresistance of TICs. Experimental approaches: RNA isolation from bulk cultures and CD44+ and CD44- cells (some of which will be inhibited for IKK/NF-κB), RT-PCR analysis to measure candidate RNAs, treatment with doxorubicin, cell death assays, tumorsphere assays. Timeline:

---Analyze the involvement of cytokines/growth factors that promote the TIC phenotype in an NF-κB-dependent manner. Experimental approaches: cytokine inhibition (neutralizing antibodies, receptor inhibitors), CD44+ cell isolation, immunoblotting, RNA analysis, tumorsphere assays.
---Explore the involvement of mTOR and Stat3 in promoting TIC phenotypes, dependent on IKK and NF-κB-dependent mechanisms. Experimental approaches: CD44+ isolation from breast cancer cells, inhibitor studies, immunoblotting, tumorsphere assays.

Statement of Work/Aim 2 (Directed by Dr. Baldwin)
---Analyze the ability of IKK (and potentially other inhibitors) to block growth of tumor xenografts derived from basal-like and Her2+ breast cancer cells, focused on effects of inhibitors on TICs. Experimental approaches: establish breast cancer cell xenograft tumors (derived from 3 breast cancer cell lines), treat xenograft tumors (10 control and 10 experimental tumors each year) with inhibitors (IKK inhibitor and potentially others derived from work in Aim 1), determine if tumor growth is inhibited potentially through induction of cell death, determine if TICs are targeted by the inhibitor as measured through TIC markers including CD44+ cells, EMT markers, and angiogenesis.
---Characterize in situ models of basal-like and Her2+ relative to responses to inhibitors and effects on TICs. Experimental approaches: C3Tag and Her2+ transgenic animal genotyping [animals are maintained in the Lineberger tumor models core facility], analyze 10 control and 10 treated animals with inhibitors described above, monitor tumor growth over two weeks of treatment with a focus on cell death analysis, effects on TICs, angiogenesis, and cell proliferation. As described above, if another inhibitor shows significant effects in Aim 1, this inhibitor will be pursued in this aim.

Statement of Work/Aim 3 (Work performed by Dr. Chodosh -- collaborator/W81XWH-12-10-0177 -Univ. Pennsylvania)
--- Using conditional transgenic mouse models for Her2/neu-driven breast cancers, characterize TGFβ and NF-κB-related signaling pathways in residual cancer cells and recurrent breast cancers. Experimental approaches: immunoblotting and immunofluorescence for markers of EMT, NF-κB activity, Akt activity, mTORC1 activity, Stat3, and IL-6 expression in primary tumors, recurrent tumors, and residual disease. We are collaborating with Dr. Chodosh on these goals.

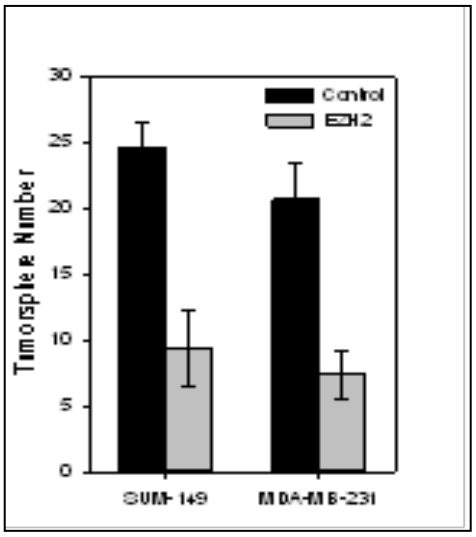
BODY (annual report):

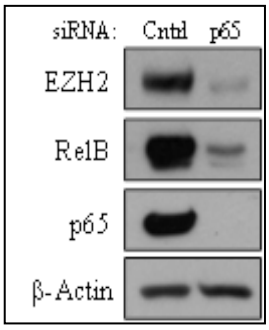
>>Regarding Aim 1/Statement of work goals:

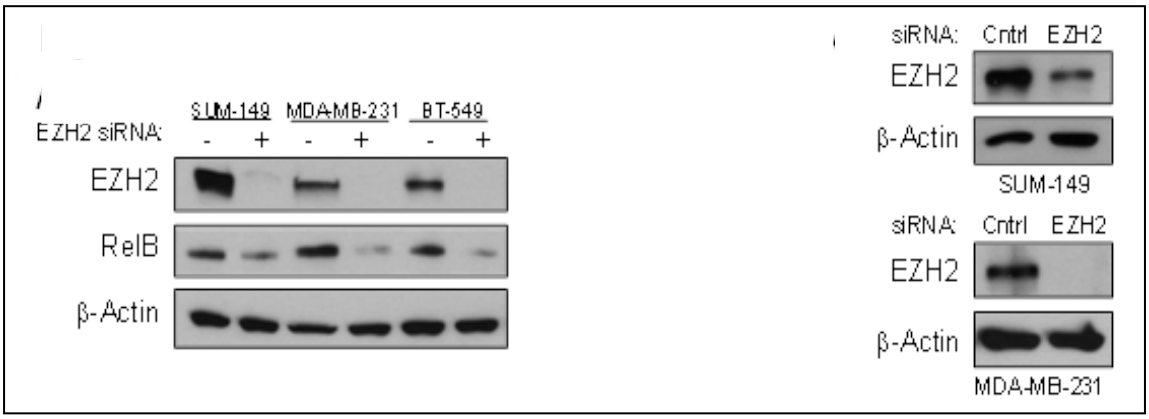
(Pt1) *Explore the involvement of NF-κB in promoting epithelial-mesenchymal transition as a regulatory mechanism in promoting TIC phenotype*. Progress: We have published (see Kendellen et al., Oncogene 2013) that both canonical and non-canonical NF-κB promote the breast cancer TIC phenotype. In Figure 5a of Kendellen (see attached) we showed that blocking NF-κB expression suppressed the EMT marker vimentin and blocking NF-κB suppressed the ability of TGFβ (known to induce EMT) to induce self-renewal (Figure 5b – Kendellen et al, attached). We showed (see that blocking canonical NF-κB (using the super-repressor form of IκBα) strongly blocked xenograft tumor growth (using a limiting dilution experiment), which is consistent with the role of NF-κB in TIC function.

(Pt2) *Determine signaling pathways, important to breast cancer TICs, that promote the EMT phenotype and the upregulation of NF-κB*. Progress: As described above, Kendellen et al (see attached) showed that both non-canonical and canonical NF-κB activation drives the TIC phenotype of breast cancer cells. For example, see Figures 2 and 3 in that publication. Figure 4f shows that TAK1 activity (an upstream marker of IKK activity in the canonical is active in the CD44+ subset of TIC cells. Figure 4d shows that non-canonical NF-κB activity, as measured in processing of p100 to p52 is higher in CD44+ cells.

In new work for this subaim of the Statement of Work, we have shown that the EZH2 pathway is important for breast cancer TICs. In the figure shown below far left, EZH2 knockdown blocks tumorsphere/self-renewal of breast cancer cells. EZH2 knockdown was approximately 80% effective (not shown). The work used two breast cancer cell lines (SUM-149 and MDA-MB-231). It was shown that EZH2 promotes breast cancer TICs (Chang et al., 2011) and that EZH2 interacts with RelA and RelB to promote gene expression in breast cancer cells (Lee et al., 2011). We have confirmed these results. We now have found that NF-κB drives the expression of EZH2 (see figure to the immediate left) – thus EZH2 may function in the TIC phenotype downstream of canonical NF-κB.







In the figure shown immediately above, knockdown of EZH2 blocks expression of RelB in basal-like and claudin-low breast cancer cell lines. This result places EZH2 upstream of RelB expression. Thus a role for EZH2 in promoting breast cancer TIC phenotype can be partly explain by the control of RelB expression.

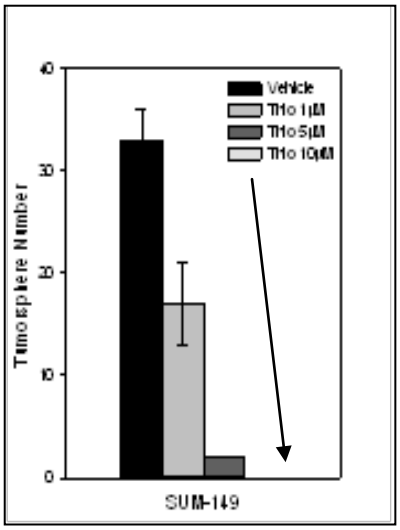
(Pt3) *Characterize the NF-κB-dependent gene expression profile in breast cancer cell TICs. Determine if NF-κB promotes chemoresistance of TICs*. Progress: We showed in Fig. 6a and 6b in Kendellen et al (see attached) that NF-κB regulates the expression of several cytokines in breast cancer cells. Important these cytokines can promote tumorspheres/self-renewal (see Fig. 6c in Kendellen et al.). We are currently studying the effects of chemotherapy treatment (cell-based) on survival of differentiated cells and on TICs. This work will be accomplished in year 2.

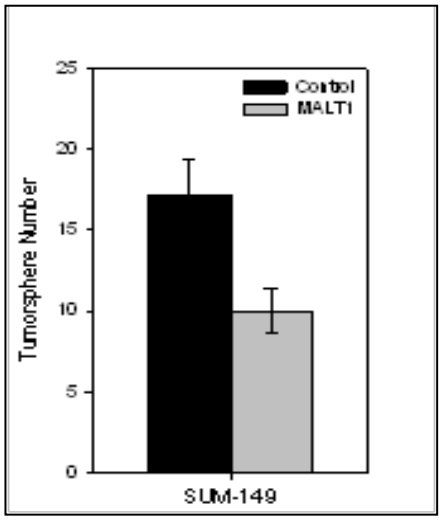
(Pt4) *Analyze the involvement of cytokines/growth factors that promote the TIC phenotype in an NF-κB-dependent manner*. Progress: As described above, Fig. 6c in Kendellen et al (attached) shows that NF-κB-regulated cytokines function to promote the TIC phenotype. In new work, we are working to show that tumor-associated macrophages promote the breast cancer TIC phenotype through secretion of cytokines (regulated by NF-κB). In vitro naïve macrophages are activated by breast cancer cells to release inflammatory cytokines. We hypothesize, based on our published work, that these cytokines will promote the TIC phenotype.

(Pt5). *Explore the involvement of mTOR and Stat3 in promoting TIC phenotypes, dependent on IKK and NF-κB-dependent mechanisms*. Progress: Cells and reagents have been generated. We will be working on these specific directions during the upcoming year.

>>Regarding Aim 2 goals:

(Pt1*). Analyze the ability of IKK (and potentially other inhibitors) to block growth of tumor xenografts derived from basal-like and Her2+ breast cancer cells, focused on effects of inhibitors on TICs.* Progress: Progress: We showed that a pharmaceutical grade IKK inhibitor (cmpdA- Bayer Pharmaceuticals) blocks the self-renewal capacity of breast cancer TICs (see Fig. 2f, Kendellen et al., attached). We have found that Thioridazine (an FDA approved drug that is known to block dopamine receptor signaling and to block MALT1, a factor upstream of IKK) strongly reduces self-renewal of breast cancer TICs. The figure at the far left shows that increasing doses of thioridazine (1μM, 5μM, and 10μM) blocks self-renewal/tumorspheres of the SUM-149 breast cancer cells. At the immediate left is a figure that shows that knockdown of MALT1 (known to be blocked by thioridazine) suppresses the TIC phenotype in SUM-149 breast cancer cells. We are currently testing the IKK inhibitor and thioridazine on breast tumor xenografts.





(Pt2). *Characterize in situ models of basal-like and Her2+ relative to responses to inhibitors and effects on TICs*. Progress: We will be analyzing two animal models to test inhibitors of IKK/NF-κB on the growth of these tumors and on the potential that they target TICs. These models are the C3Tag model for generating basal-like breast tumors and the use of patient-derived breast tumors. Both of these models are immediately available to us. Our animal protocol is now approved, and we will initiate experiments in this direction right away. Additionally, we are planning to cross the C3Tag mouse model for basal-like cancer onto mice carrying floxed alleles for IKK, RelA, and RelB to use genetic approaches to test our hypotheses. Given the results with thioridazine, we will test this compound on the two different breast tumor models.

>>Regarding Aim 3 goals.

We have provided Dr. Chodosh reagents needed to analyze the research goals under his direction. He will describe progress for this Aim in his part of the progress report.

KEY RESEARCH ACCOMPLISHMENTS:

--Previously demonstrated Her2+ positive breast cancer cells with basal-like cells, indicating differential gene expression (Merkhofer et al., 2010).

--Demonstrated that IKKα and NF-κB promote invasion in Her2+ breast cancer cells (Merkhofer et al., 2010).

--Demonstrated that NF-κB is preferentially activated in breast cancer stem cells (TICs) from basal-like breast cancer cell lines (Kendellen et al. Oncogene 2013). Showed that blocking NF-κB blocks tumor formation driven by basal-like breast cancer cells.

--Demonstrated that NF-κB functions in breast cancer TICs to promote epithelial-mesenchymal transition (Kendellen et al., 2013).

--Emerging evidence that breast cancer cells activate naïve monocytes to produce inflammatory cytokines to then further activate NF-κB in the breast cancer cells to promote TICs (unpublished).

--Evidence that EZH2 regulates RelB (non-canonical NF-κB). Thus, one mechanism of action for EZH2 in promoting breast cancer TICs is potentially through its ability to promote

REPORTABLE OUTCOMES:
--EZH2 regulates RelB to promote breast cancer TICs.

--Naïve monocytes are activated by breast cancer cells to release inflammatory cytokines to further activate NF-κB in breast cancer cells.

--MALT1 is important in the breast cancer TIC phenotype.

CONCLUSIONS: NF-κB (both canonical and non-canonical pathways) plays important roles in promoting the breast cancer tumor initiating phenotype. Inhibition of NF-κB in these cells strongly blocks tumor growth, consistent with the hypothesis that NF-κB is driving tumor formation through the TIC mechanism. Evidence indicates that inflammatory cytokines (controlled by NF-κB) promote breast cancer TICs and that tumor-associated macrophages promote the TIC phenotype. Experiments are currently focused on the mechanisms whereby NF-κB is activated in breast cancer cells, the roles that NF-κB plays in the TIC phenotype, and whether inhibiting this pathway (pharmacologically) will have a significant impact on breast tumor growth/survival.

PUBLICATIONS/MEETING ABSTRACTS:

--Merkhofer et al, 2010. Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKa. Oncogene 29, 1238-1248. See attached**.**

--Bradford, Kendellen, Baldwin. DOD Era of Hope 2011/abstract. See attached.

--Kendellen et al., *Oncogene* 2013. Demonstration that canonical and non-canonical NF-κB promote that breast cancer TIC phenotype. See attached.

PERSONNEL RECEIVING PAY:
--Albert S. Baldwin (PI)
--Cortney Lawrence (Postdoctoral Research Associate)
--Jose Roques (Research Technician)

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Oncogene (2010) 29, 1238–1248

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ORIGINAL ARTICLE

Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKα

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The membrane bound receptor tyrosine kinase Her2 is overexpressed in approximately 30% of human breast cancers, which correlates with poor prognosis. Her2-induced signaling pathways include MAPK and PI3K/Akt, of which the latter has been shown to be critical for Her2$^+$ breast cancer cell growth and survival. In addition, the NF-κB pathway has been shown to be activated downstream of Her2 overexpression; however, the mechanisms leading to this activation are not currently clear. Using Her2$^+$/ER$^-$ breast cancer cells, we show that Her2 activates NF-κB through the canonical pathway which, surprisingly, involves IKKα. Knockdown of IKKα led to a significant decrease in transcription levels of multiple NF-κB-regulated cytokine and chemokine genes. siRNA-mediated knockdown of IKKα resulted in a decrease in cancer cell invasion, but had no effect on cell proliferation. Inhibition of the PI3K/Akt pathway had no effect on NF-κB activation, but significantly inhibited cell proliferation. Our study suggests different roles for the NF-κB and PI3K pathways downstream of Her2, leading to changes in invasion and proliferation of breast cancer cells. In addition this work indicates the importance of IKKα as a mediator of Her2-induced tumor progression.
Oncogene (2010) **29,** 1238–1248; doi:10.1038/onc.2009.410; published online 30 November 2009

Keywords: Her2; IKKalpha; NF-KappaB

Introduction

The epidermal growth factor receptor Her2 is amplified in 20–30% of breast cancers, which typically do not express estrogen receptor, and are often correlated with poor prognosis and/or chemoresistance, making Her2 an important therapeutic target (Slamon *et al*., 1987, 1989; Hynes and Stern, 1994; Klapper *et al*., 2000). The Her2-specific antibody trastuzumab and the dual EGFR/Her2 inhibitor lapatinib have been shown to decrease growth of Her2-overexpressing tumors (Pegram *et al*., 1998; Baselga *et al*., 1999); however, a majority of patients treated with trastuzamab develop resistance (Slamon *et al*., 2001), indicating the importance of elucidating alternative therapeutic targets in this disease. Her2-overexpression was first shown to activate NF-κB over a decade ago (Galang *et al*., 1996), however, the role that NF-κB has in development and progression of Her2-overexpressing breast cancer is still poorly understood. In addition, the pathway leading to NF-κB activation downstream of Her2 is not well characterized.

NF-κB is an important transcription factor that has been shown to be involved in expression of genes involved in key cellular processes including innate and adaptive immunity (Bonizzi and Karin, 2004), cell proliferation and survival (Papa *et al*., 2006), lymphoid organ development (Weih and Caamano, 2003), as well as being activated in a variety of different cancers, including breast cancer (Cogswell *et al*., 2000; Basseres and Baldwin, 2006; Belguise and Sonenshein, 2007). The NF-κB family of transcription factors consists of five subunits: RelA (p65), RelB, c-Rel, p105/p50 and p100/p52. These subunits are evolutionarily conserved and exist as hetero- or homodimers (Hayden and Ghosh, 2004). The p65/p50 heterodimer is the most abundant NF-κB complex in the cell and is regulated by the so-called canonical pathway. Following stimulation with activators such as TNF, IκB is phosphorylated by the inhibitor of kappaB kinase (IKK) complex. The IKK complex consists of two catalytic subunits IKKα, and IKKβ, and a regulatory subunit IKKγ (NEMO), which binds both catalytic subunits at their NEMO-binding domain (Gilmore, 2006). In the canonical pathway, IKKβ phosphorylates IκBα leading to its degradation and NF-κB nuclear accumulation (Ghosh and Karin, 2002). Furthermore, the p65 subunit of NF-κB can be phosphorylated on multiple residues, including serine 536, which is important for transactivation potential (Sakurai *et al*., 1999). NF-κB activation can also occur via the alternative, or non-canonical pathway. Activation of NF-κB in the non-canonical pathway, most common in B cells, involves IKKα and is IκBα-independent (Solt and May, 2008). Thus most current models place IKKβ as the dominant IKK subunit in the canonical pathway with IKKα functioning in the non-canonical system. Few studies have addressed the individual roles of IKKα and IKKβ downstream of oncoprotein-dependent signaling.

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Received 6 April 2009; revised 3 September 2009; accepted 12 October 2009; published online 30 November 2009

Using an siRNA approach, we set out to determine how NF-κB is activated downstream of Her2, and what role the IKK complex has in this signaling cascade, as well as how the activation of the IKK kinases may lead to a malignant state. Although the classical pathway has long been thought to require IKKβ, here we show that IKKα has a larger role than IKKβ in the activation of NF-κB in Her2$^+$ breast cancer cells, including the phosphorylation of the p65 subunit at serine 536. Using siRNA to the IKK kinases, we show that knockdown of IKKα leads to a change in the gene expression profile in Her2$^+$ cells, including a notable cytokine and chemokine gene expression signature. Furthermore, knockdown of IKKα by siRNA led to a marked decrease in invasive ability in SKBr3 cells, yet had no effect on cell proliferation. Taken together, our data suggests that Her2 can activate NF-κB through the canonical pathway. Surprisingly, this activation occurs primarily through IKKα, a subunit typically not thought to be involved in the canonical pathway. Interestingly, we have discovered differential roles for the IKK kinases with IKKα specifically involved in an invasive oncogenic phenotype in Her2$^+$ breast cancer cells.

Results

Lapatinib inhibits Her2 activation of NF-κB and Akt

It has previously been shown that Her2-overexpression leads to activation of NF-κB family members involved in the canonical pathway, specifically the p65/p50 heterodimeric complex (Galang *et al*., 1996; Biswas *et al*., 2004). Given this result, we investigated whether the dual EGFR/Her2 inhibitor Lapatinib (Tykerb, GW572016) could block Her2-induced p65 phosphorylation at serine 536, a marker of increased NF-κB transcriptional activity (Sakurai *et al*., 1999). Five breast cancer cell lines were treated with 1 μM lapatinib for 12 h and whole-cell extracts were analysed for expression of phosphorylated p65. A marked decrease in p65 phosphorylation was observed in Her2-ovexpressing tumor cell lines (SKBr3 and MDA-MB-453) upon treatment with lapatinib, whereas non-Her2-overexpressing tumor cell lines (MCF7 and MDA-MB-231) showed no change (Figure 1a). The H16N2-Her2 cell line also showed a decrease in p65 phosphorylation upon lapatinib treatment. Overexpression of Her2 in this cell line results in NF-κB activation, as the parental cell line, H16N2-pTP, has very little basal p65 phosphorylation (Supplementary Figure 1). To further investigate how Her2 signals to NF-κB, we chose to use the tumor-derived SKBr3 cell line, as it has previously proven to be an excellent *in vitro* model for Her2$^+$/ER$^-$ breast cancer (Singh *et al*., 2007). SKBr3 cells were treated with 1 μM lapatinib or vehicle control over a course of 24 h and whole-cell extracts were analysed for levels of phosphorylated IκBα. Phosphorylation of IκBα at serines 32 and 36 was inhibited within 3 h of lapatinib treatment (Figure 1b). Stabilization of IκBα was also observed, consistent with the loss of phosphorylated IκBα. It has previously been shown that Her2-overexpression activates the PI3K/Akt pathway and that lapatinib can inhibit Akt phosphorylation in lapatinib-sensitive Her2 overexpressing breast cancer cell lines (Hegde *et al*., 2007). Similarly, we observe a decrease in phosphorylation of Akt at serine 473 in the lapatinib-sensitive SKBr3 cell line upon treatment with lapatinib (Figure 1c). This indicates that Her2 can activate both the NF-κB and the PI3K/Akt pathways, and that pharmacological inhibition of Her2 leads to subsequent inhibition of these survival pathways.

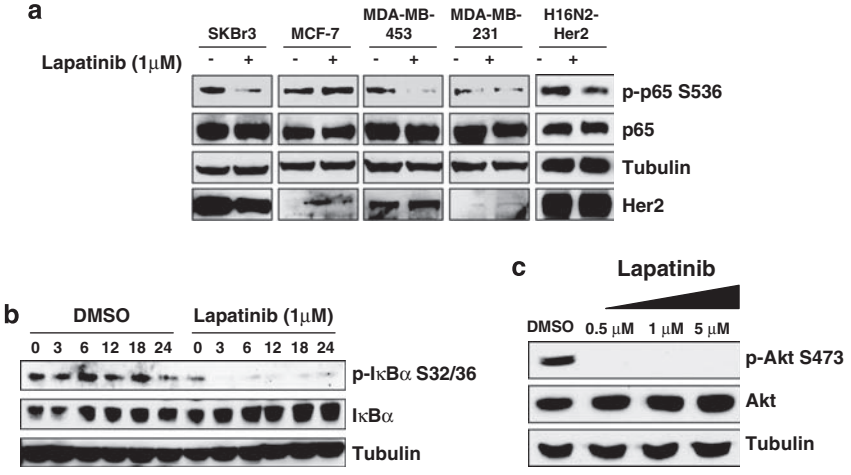

Figure 1 Lapatinib treatment inhibits the NF-κB and PI3K pathways in Her2-overexpressing cells. (**a**) Western blot of phospho-p65^{S536} in multiple breast cancer cell lines treated with lapatinib. Breast cancer cell lines were treated with 1 μM dual EGFR/Her2 inhibitor lapatinib or DMSO vehicle control for 12 h. Western blots were performed with 25 μg protein from whole-cell extracts. (**b**) Western blot of phospho-IκBα$^{S32/36}$ in SKBr3 cells treated with lapatinib. SKBr3 cells were treated with lapatinib (1 μM) or DMSO control over a course of 24 h and levels of phospho-IκBα$^{S32/36}$ were measured by western blot of 25 μg total protein from whole-cell extracts. (**c**) Western blot of phospho-Akt^{S473} in SKBr3 cells treated with lapatinib. SKBr3 cells were treated for 12 h with dual EGFR/Her2 inhibitor lapatinib and levels of phospho-Akt^{S473} were measured by western blot of 25 μg protein from whole-cell extracts.

Her2 activates the NF-κB canonical pathway through IKKα and IKKβ

We next examined the role of the IKK complex in the activation of NF-κB downstream of Her2. siRNA targeting the catalytic subunits of the IKK complex (IKKα and IKKβ) was transfected into Her2-overexpressing breast cancer cells and whole-cell extracts were analysed for markers of NF-κB activation. In the Her2-ovexpressing SKBr3, H16N2-Her2 and MDA-MB-453 cells, knockdown of IKKα led to a greater decrease in p65 phosphorylation than knockdown of IKKβ (Figure 2a). Mouse embryonic fibroblasts (MEFs) lacking IKKα, as well as wild-type cells, were transduced with Her2 wild-type and constitutively active constructs. Transduction of these constructs resulted in increased p65 phosphorylation in wild-type MEFs; however, no increase in phosphorylation was seen in IKKα −/− cells (Supplementary Figure 2). A similar result was obtained using IKKβ −/− cells (data not shown), indicating that both IKKα and IKKβ are important for Her2 to activate NF-κB in murine fibroblasts. To further investigate the role IKKα may have in the activation of classical NF-κB complexes downstream of Her2, siRNA was again used to target IKK in SKBr3 cells stably expressing a 3× κB luciferase reporter construct, as well as in H16N2–

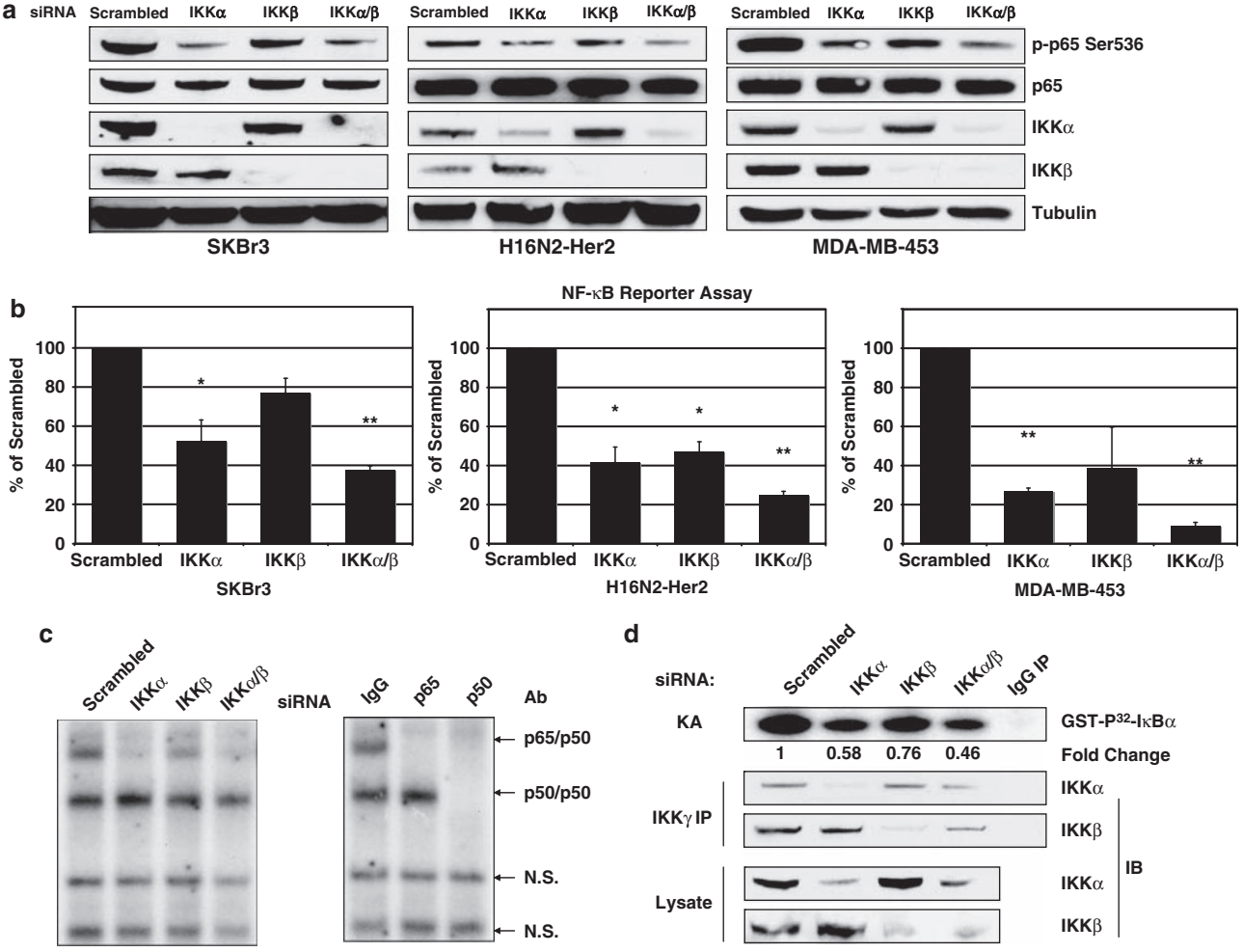

Figure 2 Her2 activation of NF-κB via IKKα and IKKβ involves the canonical pathway. (**a**) Western blot of phospho-p65^{S536} in Her2-overexpressing breast cancer cells transfected with siRNA to IKK catalytic subunits. SKBr3 (left), H16N2-Her2 (center) and MDA-MB-453 (right) cells were transfected with 100 nM siRNA to IKKα and IKKβ and whole-cell extracts were prepared after 72 h and western blot analysis performed. (**b**) NF-κB luciferase reporter assay of SKBr3, H16N2-Her2 and MDA-MB-453 cells transfected with IKK siRNA. Whole-cell extracts were prepared 72 h post-siRNA transfection and luciferase levels were measured. Statistically significant differences were determined by Student's *t*-test (* <0.05 ** <0.001). Fold change of reporter activity with IKK knockdown is shown relative to scrambled siRNA-treated cells. Values are the average of at least three experiments. Error bars are ± 1 s.e. Samples are normalized by protein concentration (SKBr3) or renilla (H16N2-Her2 and MDA-MB-453). (**c**) Electrophoretic mobility shift assay of SKBr3 cells transfected with IKK siRNA. Nuclear extracts were prepared after 72 h. Identities of the bound complexes were determined by super-shift with antibodies to p65 and p50. Non-specific binding complexes are noted with as NS (**d**) Kinase assay measuring IKK *in vitro* phosphorylation of IκBα. SKBr3 cells were transfected with IKK siRNA for 72 h and IKKγ was immunoprecipitated from 500 μg whole-cell extracts. Ability of immunoprecipitated complex to phosphorylate purified GST-IκBα was measured (KA). Amount of IKKα and IKKβ in immunoprecipitated complex (IP) and whole-cell extracts (lysate) were measured. Fold change in kinase activity was calculated using pixel densitometry and compared with scrambled siRNA-transfected cells.

Her2 and MDA-MB-453 cells transiently transfected with the 3× κB reporter plasmid. Knockdown of IKKα or a combination of IKKα and IKKβ led to a significant decrease in luciferase reporter activity (student's *t*-test $*<0.05$ and $**<0.001$, respectively), whereas knockdown of IKKβ did not show a significant decrease in luciferase reporter activity in two of the three cell lines (Figure 2b). An electrophoretic mobility shift assay was performed to further investigate the role of IKK in Her2 activation of NF-κB in SKBr3 cells. Knockdown of IKKα led to a greater decrease in NF-κB DNA-binding activity than IKKβ knockdown (Figure 2c). Supershift analysis indicated that loss of IKKα leads to a decrease in DNA binding of classical pathway NF-κB heterodimers p65/p50. Phosphorylation of IκBα by the catalytic subunits of the IKK complex is a hallmark of activation of the canonical NF-κB pathway, therefore we measured this kinase activity upon knockdown of IKKα or IKKβ. The IKK complex was immunoprecipitated with IKKγ, the scaffolding subunit of the IKK complex. Knockdown of IKKα led to a greater decrease of *in vitro* phosphorylation of IκBα than knockdown of IKKβ (Figure 2d), further indicating IKKα has a prominent role in the canonical pathway in Her2-overexpressing cells. Taken together, these results show that IKKα has a more significant role than IKKβ in the activation of the NF-κB canonical pathway in Her2-overexpressing breast cancer cells.

Knockdown of IKKα and IKKβ leads to distinct gene expression profiles

We next determined if knockdown of the two IKK catalytic subunits leads to differential changes in gene expression in Her2-overexpressing cells. A chemiluminescent oligo-based array was used to measure expression of 219 genes. Upon knockdown of IKKα or IKKβ, significant decrease in expression was seen in 14 genes (Supplementary Table 1). Genes that showed significant changes in expression upon siRNA transfection were validated by quantitative real-time PCR. Decrease in expression of pro-inflammatory cytokines and chemokines IL-6, IL-8, CCL-2, TNF and the serine-protease uPA, was greater upon siRNA knockdown of IKKα than IKKβ in both SKBr3 and H16N2-Her2 breast cancer cell lines (Figure 3a). To show that IKK-dependent changes in gene expression were occurring through modulation of NF-κB transcriptional activity, we performed RNAi against the classic subunit p65 in SKBr3 and H16N2-Her2 cells and assayed expression of mRNA by quantitative real-time PCR. Gene expression analysis showed that knockdown of p65 by siRNA led to a significant decrease in gene transcription levels of IL-8, IL-6, TNF and uPA (Figure 3b). This transcriptional profile mirrors that seen upon knockdown of IKK, specifically IKKα, suggesting that induction of chemokines and cytokines in Her2 breast cancer cells occurs through IKK activation of p65. We next measured changes in expression of these genes in SKBr3 cells following treatment with lapatinib to confirm this activation of NF-κB-regulated genes was induced downstream of overexpression of Her2. Treatment of SKBr3 cells with 1 μM of lapatinib led to a significant decrease in gene expression of IL-6, IL-8, CCL-2, TNF and uPA at both 8 and 16 h post treatment (Figure 3c). Taken together, this suggests that Her2 activates NF-κB through the canonical pathway involving IKKα and leading to an increase in multiple NF-κB-regulated genes involved in tumor progression.

Activation of NF-κB in Her2-overexpressing cells requires NEMO

The scaffolding subunit of the IKK complex, IKKγ (NEMO), is required for activation of NF-κB canonical pathway involving IKKβ (Gilmore, 2006), and inhibition of the IKK signalsome with the Nemo-binding domain peptide can block NF-κB activation (Biswas *et al*., 2004). We used an siRNA approach to determine the importance of NEMO in NF-κB activation in Her2-overexpressing cell lines. siRNA knockdown of NEMO led to a marked decrease in p65 phosphorylation in all three $Her2^+$ cell lines (Figure 4a). NF-κB luciferase reporter activity was also significantly decreased in these cell lines upon siRNA knockdown of NEMO (Figure 4b). We performed quantitative real-time PCR analysis in the SKBr3 cell line upon NEMO knockdown to determine if this resulted in a similar gene expression profile as IKKα knockdown. Consequently, IL-6, IL-8, TNF and CCL2 all showed a significant decrease in expression upon NEMO knockdown, though uPA expression levels did not change (Figure 4c). To rule out any effect loss of IKKα could have on non-classical activation of NF-κB, we analysed processing of the p100 subunit. Cleavage of the precursor NF-κB protein p100 to p52 is a hallmark of activation of the non-canonical pathway. No significant effect was seen on p100 processing to p52 upon knockdown of either of the IKK subunits in $Her2^+$ cells (Figure 4d). These results suggest that NF-κB activation in $Her2^+$ cells occurs through IKKα and this requires the NEMO subunit. In addition, these results indicate that the non-canonical NF-κB-signaling pathway is not activated in $Her2^+$ breast cancer cells.

Activation of the NF-κB canonical pathway is independent of the PI3K pathway

It was reported earlier that expression of dominant-negative PI3K and Akt plasmids can block NF-κB DNA binding downstream of Her2 (Pianetti *et al*., 2001). Therefore, we investigated if NF-κB activation downstream of Her2 is dependent on the PI3K/Akt pathway. Upon treatment of SKBr3 cells with lapatinib, phosphorylation of Akt at Serine 473 decreases dramatically (Figure 1a). Treatment with the PI3K inhibitor LY294002 also blocked phosphorylation of Akt at serine 473, however, LY294002 had no effect on the phosphorylation status of p65 at serine 536 in SKBr3, H16N2-Her2 or MDA-MB-453 cells (Figures 5a–c). Furthermore, treatment of SKBr3 cells stably expressing the 3× κB luciferase reporter with LY294002 had no

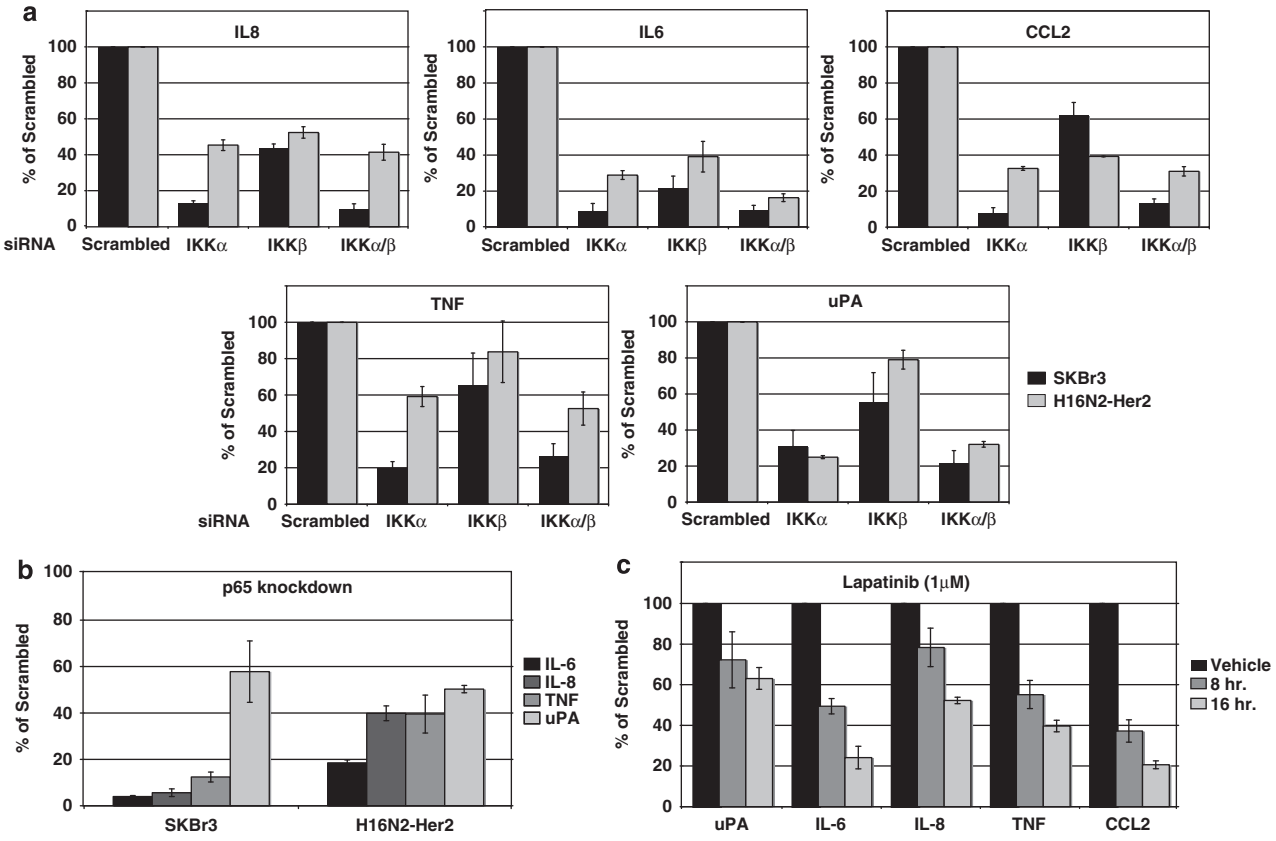

Figure 3 Her2 induces NF-κB-regulated gene expression through IKKα and IKKβ. (**a**) Quantitative real-time PCR of multiple genes shows different gene expression profiles upon IKKα or IKKβ knockdown. qRT–PCR was performed on extracts from SKBr3 (black bars) and H16N2-Her2 (gray bars) cells transfected with 100 nM IKKα or IKKβ siRNA for 72 h. Gene expression levels were normalized to Gus or GAPDH and presented as fold change versus cells transfected with scrambled control siRNA. Values are the average of at least three experiments. Error bars are ± 1 s.e. (**b**) Quantitative real-time PCR of multiple genes upon knockdown of p65 by siRNA. SKBr3 and H16N2-Her2 cells were transfected with 100 nM siRNA for 72 h and gene expression levels were measured. Fold change of transcript levels is shown relative to scrambled siRNA-treated cells. Values are the average of at least three experiments. Error bars are ± 1 s.e. (**c**) Quantitative real-time PCR shows inhibition of Her2 by lapatinib blocks NF-κB-regulated gene expression. SKBr3 cells were treated with 1 μM lapatinib for 8 or 16 h and gene expression levels of uPA, IL-6, IL-8, TNF and CCL2 were compared with DMSO-treated cells. Fold change of transcript levels is shown relative to scrambled siRNA-treated cells. Error bars are ± 1 s.e.

effect on NF-κB transcriptional activity (Figure 5d). These results show that Her2 activates Akt through PI3K, and that the Her2-induced activation of NF-κB is independent of this pathway.

IKKα induces cell invasion but not cell proliferation

Having determined that overexpression of Her2 leads to IKKα-dependent activation of the NF-κB classical pathway, we next sought to determine how this signaling may promote oncogenic phenotypes. We investigated the effect IKK activation may have on proliferation of Her2-overexpressing breast cancer cells. SKBr3 cells were transfected with siRNA to the IKK catalytic subunits and cell proliferation was measured by MTS assay. Knockdown of IKKα or IKKβ had no inhibitory effect on cell proliferation (Figure 6a). As a control, SKBr3 cells were treated with the PI3K-inhibitor LY294002, as well as lapatinib. Inhibition of PI3K/Akt or Her2 led to a dramatic decrease in cell growth (Figure 6b), consistent with what has been previously reported, suggesting that Her2 drives cell proliferation through the PI3K/Akt pathway. Our previous results have shown IKK/NF-κB dependent increases in proinflammatory cytokines downstream of Her2, and these genes have been shown to promote increased motility and invasiveness. Furthermore, overexpression of Her2 has been shown to lead to increase in invasiveness of breast cancer cells (Arora *et al*., 2008). We reasoned that NF-κB activity downstream of Her2 may contribute to increased invasiveness of Her2 breast cancer. To address this question, SKBr3 cells were transfected with siRNA to IKKα and IKKβ and the ability of the cells to invade through a basement membrane was measured. Knockdown of IKKα led to a significant decrease in invasiveness of SKBr3 cells while knockdown of IKKβ had no effect (Figure 6c). This suggests that Her2 overexpression results in the activation of at least two independent oncogenic signaling pathways, one involving PI3K/Akt and another involving NF-κB, which have two different but important roles in promoting tumorigenesis (Figure 6d).

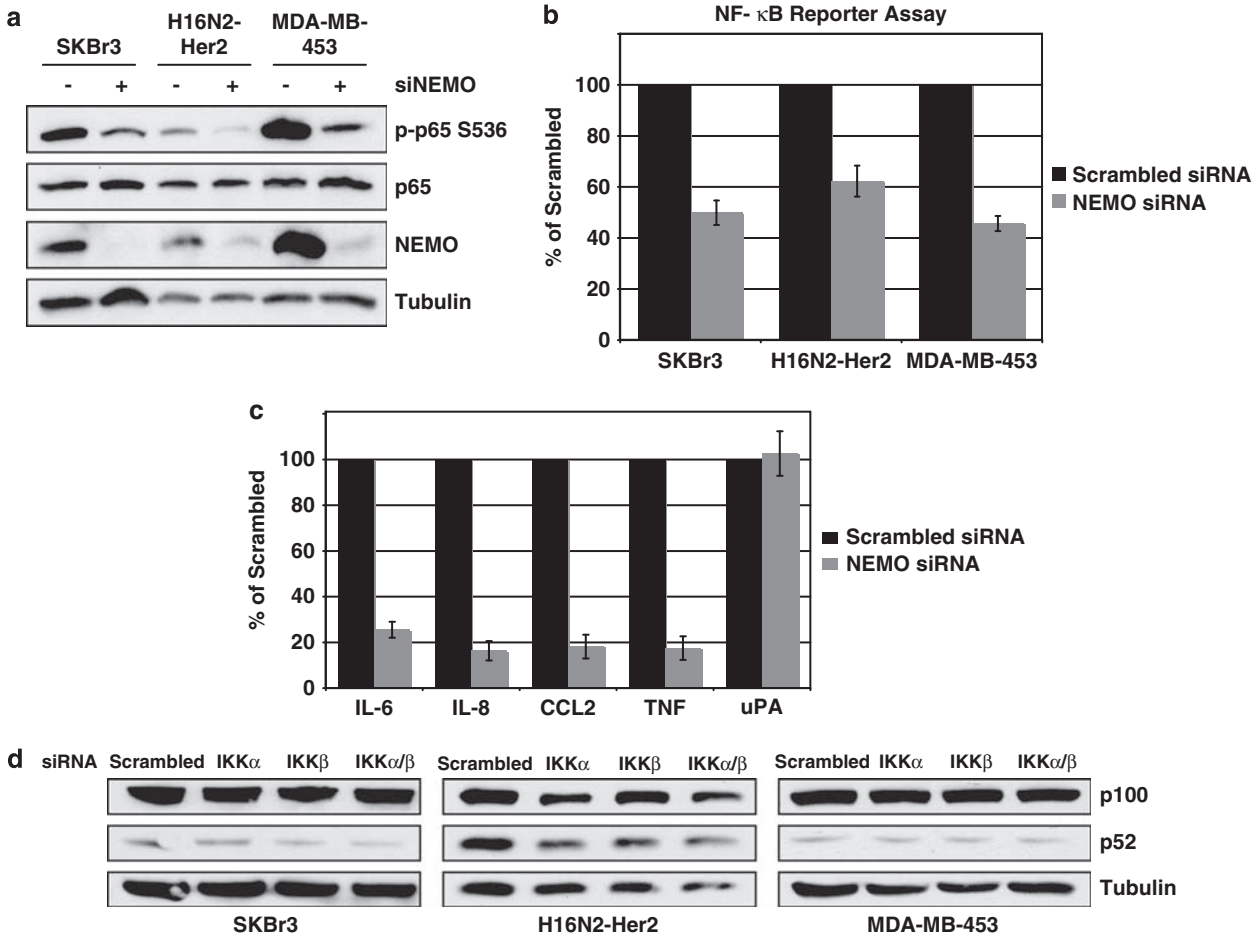

Figure 4 Knockdown of NEMO blocks NF-κB activation through the canonical pathway. (**a**) Her2$^+$ breast cancer cells were transfected with 100 nM NEMO siRNA and whole-cell lysates were collected 72 h post transfection and western blot analysis of phosphorylated p65 was performed using 25 μg total protein. (**b**) Her2$^+$ cell lines were transfected with 100 nM NEMO siRNA and whole-cell extracts were prepared 72 h post-siRNA transfection and luciferase levels were measured. Fold change of reporter activity with IKK knockdown is shown relative to scrambled siRNA-treated cells. Values are the average of at least three experiments. Error bars are ± 1 s.e. Samples are normalized by protein concentration (SKBr3) or renilla (H16N2-Her2 and MDA-MB-453). (**c**) SKBr3 cells were transfected with 100 nM NEMO siRNA and extracts were isolated after 72 h and qRT–PCR was performed. Fold change of transcript levels is shown relative to scrambled siRNA-treated cells. Error bars are ± 1 s.e. (**d**) Her2-overexpressing breast cancer cells were transfected with 100 nM siRNA to IKKα or IKKβ and whole-cell extracts were collected 72 h post transfection. Levels of p100 and p52 were measured by western blot analysis using 25 μg of total protein.

Discussion

Although Her2-positive breast cancer is known to activate both NF-κB and PI3K/Akt pathways, (Pianetti *et al*., 2001; Knuefermann *et al*., 2003; Biswas *et al*., 2004; She *et al*., 2008), it has been unclear how Her2 induces NF-κB and whether PI3K is involved with this pathway. In addition, potential roles for IKKα and IKKβ in controlling Her2-induced NF-κB have not been addressed. The latter point is of interest because IKKα and IKKβ have previously been associated with controlling distinct NF-κB pathways, with IKKβ controlling the so-called canonical pathway and IKKα controlling the non-canonical pathway. These issues are potentially quite important in the therapeutic setting. Our data indicate the following: (i) IKKα has an important role in controlling the ability of Her2 to activate NF-κB through the canonical pathway (including phosphorylation of IκBα, phosphorylation of RelA/p65, activation of IKK and regulation of gene expression), (ii) IKKα controls the invasion of Her2$^+$ cells, with apparently little contribution of IKKβ in this process and (iii) PI3K-dependent pathways do not contribute to the direct activation of NF-κB in these cells.

Previous experiments from several groups have shown that IKKβ has a major role in controlling canonical NF-κB activation downstream of inflammatory cytokines such as TNF (Verma *et al*., 1995). The potential contribution of IKKα to NF-κB activation downstream of Her2-dependent signaling or to that induced by other oncoproteins has not been fully elucidated. Lapatinib has been shown to be effective in its inhibition of the Akt and Erk pathways in Her2 overexpressing breast cancer cell lines and human tumor xenografts, but there are no reports of it having an effect on the NF-κB pathway (Xia *et al*., 2002; Zhou *et al*., 2004), although Herceptin has been shown to inhibit NF-κB activation

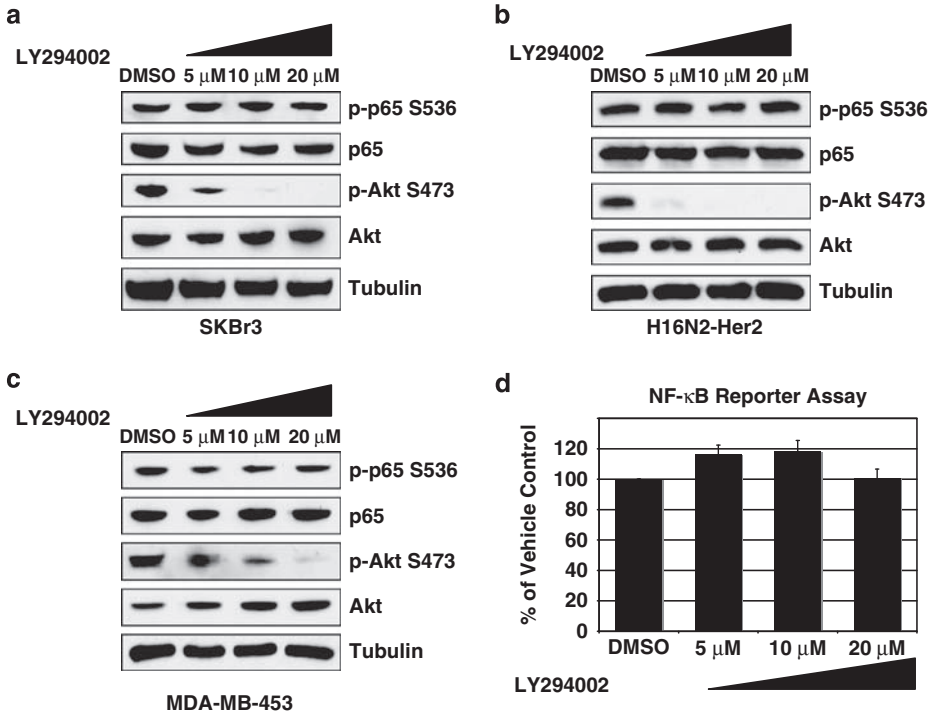

Figure 5 Inhibition of the PI3K-pathway does not block NF-κB activation. Western blot of phospho-p65 serine 536 from SKBr3 (**a**), H16N2-Her2 (**b**) and MDA-MB-453 (**c**) cells treated with PI3K-inhibitor inhibitor LY294002 for 2 h. Western blot analysis was performed with 25 μg whole-cell extracts. (**d**) Luciferase reporter assay of SKBr3 cells were treated with LY294002 overnight. Fold change of reporter activity with PI3K-inhibitor treatment is shown relative to vehicle-treated cells. Values are the average of at least three experiments. Error bars are ± 1 s.e. Samples are normalized by protein concentration.

in SKBr3 cells (Biswas *et al*., 2004). In our studies, treatment of Her2-overexpressing cell lines with 1 μM lapatinib led to a marked decrease in phosphorylation of NF-κB subunit p65 at serine 536 and of IκBα at serines 32 and 36 (Figures 1a and b). Lapatinib also blocked NF-κB-induced gene transcription (Figure 3c). Treatment of SKBr3 cells with lapatinib led to complete loss of phosphorylation of Akt at serine 473 (Figure 1c), a marker for Akt activation.

To address potential contributions of IKKα and IKKβ to NF-κB activation in $Her2^+$ cells and to the oncogenic phenotype, we used an IKK knockdown approach in Her2-overexpressing cells. Knockdown of IKKα led to a more dramatic reduction in p65 phosphorylation at Ser536 than did knockdown of IKKβ (Figure 2a). Furthermore, knockdown of IKKα strongly reduced NF-κB activation as measured through electrophoretic mobility shift assay and NF-κB-dependent reporter assays, whereas IKKβ knockdown had less of an effect (Figures 2b and c). Similarly, knockdown of IKKα was more effective at blocking IKK activity than knockdown of IKKβ (Figure 2d). SKBr3 cells show low levels of p52/NF-κB2, which is derived from IKKα-dependent processing of the p100/NF-κB2 precursor. Knockdown of IKKα had little effect on p52 levels in these cells, indicating that the non-canonical pathway does not appear to be active in SKBr3 cells at a measurable level. Consistent with this, very low to undetectable levels of p52 or RelB are detected in the nuclei of SKBr3 cells (data not shown). It is important to note that inhibition of IKKβ can lead to a compensatory response whereby IKKα controls canonical NF-κB activation in some cell types (Lam *et al*., 2008). Our studies clearly indicate that loss of IKKα leads to reduced NF-κB activation downstream of Her2-induced signaling. A study showing that IKKα is necessary for self-renewal of Her2-transformed mammary-initiating tumor cells (Cao *et al*., 2007) is consistent with our results showing the importance of IKKα in controlling NF-κB downstream of Her2. The way in which Her2 may selectively activate IKKα in breast cancer remains to be investigated. One possibility is selective activation of IKKα by the kinase NIK, as NIK has been shown to associate with ErbB2 family member EGFR (Habib *et al*., 2001) and has been shown to be recruited to EGF/heregulin receptor-signaling complexes (Chen *et al*., 2003).

The knockdown studies were extended to analysis of NF-κB-dependent target gene expression (Figure 3a). Knockdown of IKKα lead to a more dramatic reduction in gene expression of IL-6, IL-8, CCL2, TNF and uPA than did knockdown of IKKβ. Decreased expression of these genes upon knockdown of the p65 subunit of NF-κB indicates that this activation is occurring through the canonical pathway (Figure 3b). To show that these genes are controlled through Her2, and not through Her2-independent pathways, lapatinib was shown to block target gene expression (Figure 3c). This increase in chemokine and cytokine gene expression by Her2, as well as the increase in the expression of the

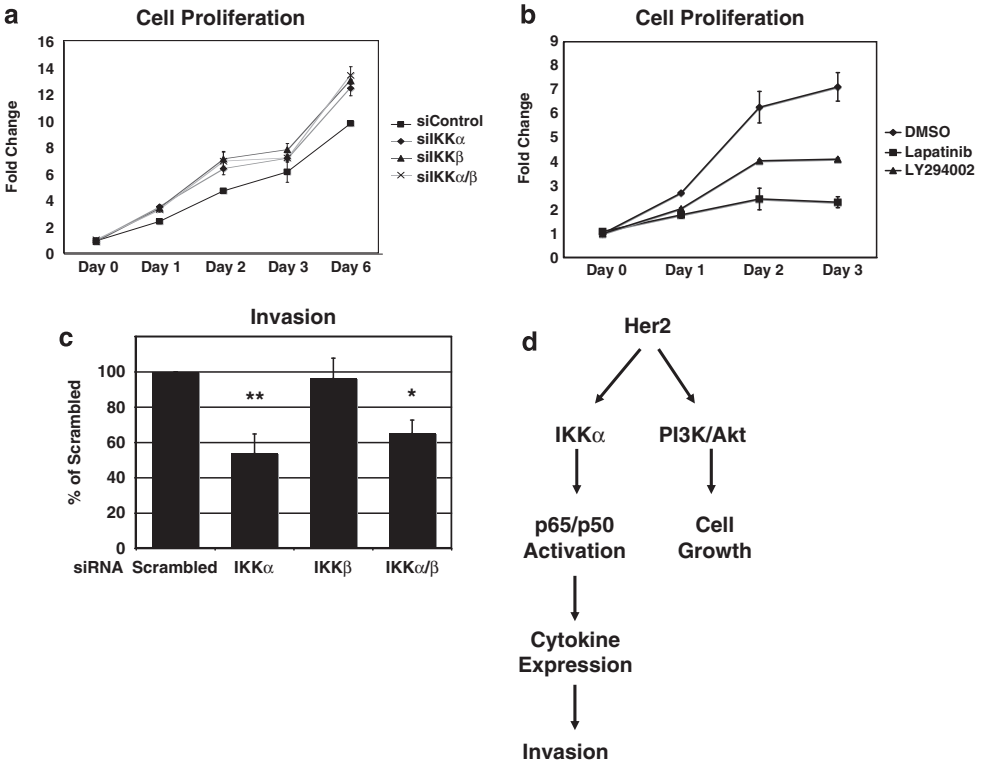

Figure 6 Inhibition of PI3K blocks cell proliferation, knockdown of IKKα blocks cell invasion. (**a**) Cell proliferation of SKBr3 cells transfected with siRNA to IKKα or IKKβ was measured for 6 days post-transfection compared with scrambled siRNA-treated cells using CellTiter cell viability reagent. Knockdown of IKK by siRNA led to a slight increase in cell proliferation. Error bars represent ±1 s.d. (**b**) Cell proliferation of SKBr3 cells treated with PI3K inhibitors LY294002 (10 μM) or EGFR/Her2 inhibitor lapatinib (1 μM) was measured over 3 days. Both inhibitors showed a significant decrease in cell proliferation over a course of 3 days. Error bars represent ±1 s.d. (**c**) SKBr3 cells were transfected with 100 nM siRNA to IKKα or IKKβ and cell invasion was measured after 48 h fluorometrically. Statistical significance was measured by Student's *t*-test (*<0.01, **<0.001). Error bars represent ±1 s.d. (**d**) Model of activation NF-κB and PI3K/Akt pathways downstream of Her2 overexpression.

serine protease uPA, shows a large similarity to Her2-induced gene expression signatures, which have been previously reported, and this increase has been implicated in progression of multiple different cancers, including breast cancer (Wang *et al*., 1999; Arihiro *et al*., 2000; Chavey *et al*., 2007; Vazquez-Martin *et al*., 2008). Therefore, our gene expression data suggests that IKKα has an important role in regulating genes involved in breast cancer progression, and this requires the scaffolding subunit NEMO (Figure 4).

Some studies indicate that NF-κB can be activated downstream of PI3K/Akt (Makino *et al*., 2004; Dan *et al*., 2008). However, experiments using the PI3K inhibitor LY294002 indicate that NF-κB is not activated in Her2$^+$ cells downstream of PI3K (Figure 5). Thus, this pathway is not a link between Her2, IKKα and NF-κB activation. We cannot rule out a PI3K-independent Akt-controlled pathway in NF-κB activation. In addition, we cannot rule out that PI3K and/or Akt have effects on NF-κB-target gene expression that function separately from the induction of NF-κB activation as assayed through experiments described above. Future studies will address Her2-regulated pathways that lead to activation of IKK. Other studies (Dillon *et al*., 2007) as well as our own (Figure 6b) show that activation of the PI3K pathway has an important role in cell proliferation/viability. Interestingly, knockdown of IKKα or IKKβ subunits (individually or together) by siRNA has no measurable inhibitory effect on cell proliferation (Figure 6a).

To determine if IKKα or IKKβ controls other oncogenic phenotypes, we used siRNA treatment and measured cell invasion of SKBr3 cells. Her2 overexpression has been shown to induce cell invasion, consistent with its ability to promote upregulation of genes such as IL-8 and uPA (Gum *et al*., 1995; Vazquez-Martin *et al*., 2008). Knockdown of IKKα, but not knockdown of IKKβ, significantly blocks the invasive phenotype of SKBr3 cells (Figure 6c). This result is consistent with the regulation of target genes by IKKα that are associated with invasive phenotype. Interestingly, other factors have linked breast cancer invasion and NF-κB, including microRNAs (Ma *et al*., 2007). MicroRNAs have been shown to negatively regulate NF-κB activity and gene expression, such as microRNA-146, which can suppress expression of IL-6 and IL-8 through a reduction in levels of IRAK1 and TRAF6 in MDA-MB-231 cells, leading to the metastatic phenotype (Bhaumik *et al*., 2008).

This study shows that Her2 activation of NF-κB requires IKKα, and this PI3K-independent activation leads to an increase in cytokine and chemokine

expression, as well as an increase in invasive phenotype (Figure 6d). These data suggest that targeting multiple pathways in Her2$^+$ breast cancer may be advantageous for effective therapy, and development of inhibitors of IKKα or the use of dual IKKα/IKKβ inhibitors may prove therapeutic in Her2$^+$ cancer cells.

Materials and methods

Cell culture and reagents
The tumor-derived SKBr3 cell lines were maintained in McCoy's 5A medium (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin. The tumor-derived MCF7, MDA-MB-453 and MDA-MB-231 cell lines, as well as MEF cell lines, were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 100 units/ml penicillin/streptomycin. The human mammary epithelial cell lines (H16N2–pTP and H16N2–Her2) were maintained as previously described (Ethier *et al*., 1993). The stable 3 × κB luciferase SKBr3 cell line was established by transfection of a luciferase reporter construct containing tandem NF-κB-binding sites from the MHC class I promoter region into SKBr3 cells with Fugene (Roche, Indianapolis, IN, USA) and maintained under selection with G418 (Geneticin, Sigma-Aldrich, St Louis, MO, USA). The Her2 wild-type and mutant (V654E) plasmids were constructed previously (Li *et al*., 2004) (Addgene plasmid 16257 and Addgene plasmid 16259, Addgene, Cambridge, MA, USA). The Her2 coding sequences were subcloned into retroviral pLHCX vector (Stratagene, La Jolla, CA, USA) and virus was produced in 293T cells with cotransfection of AmphoPAK. MEFs were transduced with virus with polybrene and lysed 48 h later. The following antibodies were purchased from commercial sources: antibodies against phospho-p65 (Ser^{536}), phospho-Akt (Ser^{473}), Akt, phospho-IκBα (Ser$^{32/36}$) and IκBα from Cell Signaling Technology (Beverly, MA, USA); antibodies against Her2, IKKα clone 14A231 and IKKβ clone10AG2 and p100/p52 from Millipore (Billerica, MA, USA), antibodies against p65 and p50 (supershift), β-tubulin and IKKγ from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibody against total p65 from Rockland (Gilbertsville, PA, USA) LY294002 and Wortmannin were purchased from Cell Signaling Technology. Lapatinib (GW572016; Tykerb, GSK, Brentford, Middlesex, UK) was a gift from Dr H Shelton Earp (University of North Carolina at Chapel Hill).

Immunoblots
Whole-cell extracts were prepared on ice with Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions supplemented with protease inhibitor mix (Roche) and phosphatase inhibitor mix (Sigma). Nuclear and cytoplasmic extracts were prepared as previously described (Mayo *et al*., 1997). Protein concentrations were determined by Bradford assay (Biorad Laboratories, Hercules, CA, USA) and SDS–PAGE analysis was performed as previously described (Steinbrecher *et al*., 2005).

Small RNA interference
The following small interfering RNAs (siRNA; siGenome SMARTpool) were obtained from Dharmacon (Lafayette, CO, USA) as a pool of four annealed double-stranded RNA oligonucleotides: IKKα (M-003473-02), IKKβ (M-003503-03), NEMO (M-003767-02), RelA (p65) (M-003533-02) and non-targeting control no. 3 (D001201-03). Cells were grown to approximately 50% confluency and transfected with 100 nmol/l siRNA with Dharmafect 1 reagent according to the manufacturer's instructions.

Quantitative real-time PCR
Total RNA extracts were obtained from cells approximately 72 h post-transfection by Trizol (Invitrogen) extraction. Two micrograms of RNA were reverse transcribed using random primers and MMLV-reverse transcriptase (Invitrogen). Real-time PCR was performed and analysed as previously described (Steinbrecher *et al*., 2005) using Taqman Gene Expression Assay primer-probe sets IL-6 (Hs00174131_m1), IL-8 (Hs001741103_m1), CCL2 (Hs00234140_m1), TNF (Hs99999043_m1) and uPA (Hs00170182_m1).

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay and NF-κB supershift analysis were done on nuclear extracts as previously described (Steinbrecher *et al*., 2005) using ^{32}P-labeled oligonucleotide probe corresponding to an NF-κB site within the MHC class I promoter region.

IKK kinase assay
Whole-cell lysates were prepared on ice for 45 min in lysis buffer containing 20 mmol/l Tris (pH 8.0), 500 mmol/l NaCl, 0.25% Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 1 × protease inhibitor (Roche Applied Science) and 1 × phosphatase inhibitor cocktail (Sigma-Aldrich). IKK complexes were immunoprecipitated from 500 μg total protein extract using IKKγ antibody (Santa Cruz Biotechnology). An *in vitro* kinase assay was done and analysed as previously described (Steinbrecher *et al*., 2005) using GST–IκBα as a substrate.

Luciferase assay
SKBr3 cells stably expressing the 3 × κB plasmid were plated in equal number in triplicate in 24-well plates and transfected with siRNA for 72 h or treated overnight with LY294002. Cells were lysed in MPER and luciferase activity was measured with Promega Luciferase Assay System (Promega, Madison, WI, USA). Luciferase levels were normalized by protein concentration using a Bradford assay. H16N2–Her2 and MDA-MB-453 cells were transfected with siRNA 72 h before lysates were obtained, and were transfected with 3 × κB reporter plasmid and pRL–CMV (Promega) renilla plasmid 24 h before lysate collection. Lysates were collected as mentioned above and luciferase levels were normalized to renilla.

Cell invasion assay
Innocyte Cell Invasion Assay Kit was purchased from Calbiochem (San Diego, California, CA, USA). Cells were transfected with siRNA for 48 h before seeding. Invasion assay was performed as per the manufacturer's protocol for 48 h. The number of invading cells was measured fluorometrically with Calcein AM.

Cell proliferation assay
Cell proliferation assay was performed as previously described (Wilson and Baldwin, 2008). Cells were cultured in the presence or absence of inhibitors, or transiently transfected

with siRNA to IKK subunits and measured at the indicated time points post-transfection.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank William Comb for his assistance in the preparation of this paper, as well as Dr H Shelton Earp and Dr Carolyn Sartor for generously providing reagents. This work is funded by NIH RO1CA73756 and RO1CA75080, and Department of Defense Grant BC074027. Support is also provided by the Samuel Waxman Cancer Research Foundation.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Oncogene (2013), 1–9

www.nature.com/onc

ORIGINAL ARTICLE

Canonical and non-canonical NF-κB signaling promotes breast cancer tumor-initiating cells

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Tumor-initiating cells (TICs) are a sub-population of cells that exhibit a robust ability to self-renew and contribute to the formation of primary tumors, the relapse of previously treated tumors and the development of metastases. TICs have been identified in various tumors including those of the breast, and are particularly enriched in the basal-like and claudin-low subtypes of breast cancer. The signaling pathways that contribute to the function and maintenance of TICs are under intense study. We explored the potential involvement of the nuclear factor-κB (NF-κB) family of transcription factors in TICs in cell lines that are representative of basal-like and claudin-low breast cancer. NF-κB was found to be activated in breast cancer cells that form tumorspheres efficiently. Moreover, both canonical and non-canonical NF-κB signaling is required for these cells to self-renew *in vitro* and to form xenograft tumors efficiently *in vivo* using limiting dilutions of cells. Consistent with this fact, canonical and non-canonical NF-κB signaling is activated in TICs isolated from breast cancer cell lines. Experimental results indicate that NF-κB promotes the function of TICs by stimulating epithelial-to-mesenchymal transition and by upregulating the expression of the inflammatory cytokines interleukin-1β and interleukin-6. The results suggest the use of NF-κB inhibitors for clinical therapy of certain breast cancers.

Oncogene advance online publication, 11 March 2013; doi:10.1038/onc.2013.64

Keywords: NF-κB; basal-like breast cancer; tumor-initiating cells; EMT; IL-6; IL-1β

INTRODUCTION

Tumors are comprised of a heterogeneous population of cells including bulk epithelial tumor cells, inflammatory cells and sub-population of cells termed cancer stem cells or tumor-initiating cells (TICs).[1] The primary characteristic of TICs is their ability to self-renew, which is measured *in vitro* by the formation of spheroid cellular structures termed tumorspheres.[2,3] In addition, TICs exhibit elevated motility and invasiveness *in vitro* that correlates with high metastatic potential *in vivo*,[4–6] and are frequently radio-[7,8] and chemo-resistant.[9,10] Importantly, TICs are thought to drive the progression of primary tumors, promote tumor recurrence and stimulate the development of metastases at distant sites.[4,5] The importance of TICs in the clinical outcome of breast cancer is evidenced by the observation that an increase in their abundance following initial systemic treatment correlates with worse prognosis.[11] TICs have been observed in multiple subtypes of human breast cancer[12] and are particularly enriched in the basal-like and claudin-low subtypes.[12–14]

The nuclear factor-κB (NF-κB) family of transcription factors contains five members, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52.[15,16] In most cells, NF-κB proteins exist as hetero- and homodimers in the cytoplasm bound to a class of inhibitory proteins called IκBs (inhibitor of κB). In response to a wide variety of cellular stimuli, NF-κB becomes active through one of two pathways. In the canonical pathway, NF-κB activation depends on the IκB kinase complex (IKK), which contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ or NEMO. Upon stimulation, IκBα is phosphorylated at Ser32/36 by IKK in a manner that requires IKKβ, which results in the degradation of IκBα and the release of the p65-p50 dimer to accumulate in the nucleus.[15] Phosphorylation of p65 at Ser536 by IKK is also important for its activity.[17] Separately, the non-canonical NF-κB pathway is regulated by an IKKα homodimer. In this cascade, RelB-p100 heterodimers are processed to RelB-p52 heterodimers in a manner that depends on IKKα. In the nucleus, NF-κB dimers activate genes including those involved in cell cycle regulation (for example, cyclin D1), suppression of apoptosis (for example, Bcl-2 and Bcl-xL) and inflammation (for example, cytokines such as interleukin (IL)-6 and IL-8).[15]

Activation of NF-κB is strongly associated with oncogenesis, as it is known to promote the oncogenic phenotype through processes including cell proliferation, inflammation, cell invasion and suppression of apoptosis.[18,19] Consistent with this fact, both canonical and non-canonical NF-κB signaling is activated in human breast cancer cell lines and primary breast tumors.[20–24] Recently, IKK/NF-κB was shown to be important in TICs isolated from HER2+ breast cancer.[25,26] Others have observed that NF-κB functions to promote proliferation in basal-like breast cancer cells.[27] Here, we have explored a potential role for NF-κB in TIC function in cells derived from basal-like and claudin-low breast cancer cells. Specifically, we show that NF-κB signaling is more highly activated in breast cancer cell lines that undergo efficient self-renewal. Moreover, inhibition of either canonical or non-canonical NF-κB signaling blunts the self-renewal of human breast cancer cells *in vitro*. Inhibition of NF-κB also reduces the formation of xenograft tumors in the mammary fat pads of nude mice *in vivo*. Mechanistically, we provide evidence that NF-κB promotes the function of TICs through stimulation of epithelial-to-

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Received 1 May 2012; revised 20 December 2012; accepted 23 December 2012

mesenchymal transition (EMT) and the production of inflammatory cytokines that are encoded by NF-κB target genes. Collectively, these data demonstrate that canonical and non-canonical NF-κB signaling have critical roles in the function of TICs derived from basal-like and claudin-low subtypes of breast cancer cells.

RESULTS

NF-κB signaling is preferentially activated in tumorsphere-forming breast cancer cells

Cell lines representing the basal-like subtype (SUM149) and claudin-low subtype (MDA-MB231) of breast cancer were utilized to investigate the role of NF-κB in the function of TICs. A hallmark of breast cancer TICs is the ability to drive the formation of spheroid structures termed tumorspheres (or mammospheres) in serum-free culture, which reflects the ability of these cells to self-renew *in vitro* (reviewed in Charafee-Jauffret *et al.*[2] and Pastrana *et al.*[28]). It was observed that both SUM149 and MDA-MB231 cells efficiently form tumorspheres over at least three cycles of culture (Figure 1a). It was then determined whether the ability of basal-like and claudin-low cancer cells to form tumorspheres correlates with the level of basal NF-κB activation in the bulk population. Importantly, both p65 and IκBα are preferentially phosphorylated in SUM149 and MDA-MB231 cells that form tumorspheres efficiently compared with MCF10A cells that form tumorspheres less efficiently (Figures 1a and b).[29]

Canonical NF-κB signaling is required for basal-like breast cancer cells to efficiently self-renew *in vitro*

To inhibit NF-κB, SUM149 cells were stably infected with a retrovirus expressing either an empty vector or IκBα-SR (a modified form of IκBα that cannot be phosphorylated) and selected with puromycin (Figure 2a). To assay for self-renewal, 100 SUM149 cells expressing empty vector or IκBα-SR were plated in serum-free media on low-adhesion plates and the number of tumorspheres were determined 5 days later. Importantly, cells in which NF-κB was inhibited by expression of IκBα-SR formed threefold fewer tumorspheres than control cells, expressing an empty vector (Figure 2b). To confirm these results, an RNA interference knockdown approach was utilized. SUM149 cells were stably infected with an empty lentiviral vector, a lentivirus encoding a scrambled short hairpin RNA (shRNA) or a lentivirus encoding a shRNA construct targeting p65 or IKKβ (Figure 2c). Subsequently, self-renewal of these cells was assayed through tumorsphere formation as described above. Importantly, knockdown of either p65 or IKKβ resulted in a statistically significant, approximately fivefold reduction in the ability of SUM149 cells to form tumorspheres (Figure 2d). Notably, expression of IκBα-SR, or knockdown of IKKβ or p65 does not alter the growth of SUM149 cells as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Supplementary Figures 1a and b). In addition, further analysis demonstrated that tumorspheres were arising from single cells (data not shown). This indicates that any variation in the formation of tumorspheres is due to reduced self-renewal, rather than an inhibition of cell growth or cytotoxicity. Interestingly, shRNA-based knockdown of IKKα (Figure 2c) induced a statistically significant, approximately fivefold reduction in the self-renewal of SUM149 cells (Figure 2d) without altering overall cell growth (Supplementary Figure 1b). Treatment with an IKKβ inhibitor (compound A[30]) blocked both basal and tumor-necrosis factor-α-induced phosphorylation of p65, (Figure 2e) and led to a reduction in tumorsphere formation that was very similar to the results obtained with shRNA knockdown of different IKK and NF-κB subunits (Figure 2f). These data demonstrate that canonical NF-κB signaling, driven by IKK, promotes self-renewal in basal-like breast cancer cells (also see below).

In addition to the breast cancer cells analyzed above, we analyzed the role of NF-κB in the claudin-low representative cell line SUM159 (reviewed in Prat and Perou[31] and Prat *et al.*[32]). Notably, similar results were obtained in SUM159 cells compared with SUM149 cells. Specifically, inhibition of IKK with compound A blocked NF-κB activity as measured by inhibition of phosphorylation of p65 and IκBα (Supplementary Figure 2a). In addition, and consistent with the findings using basal-like breast cancer cells, compound A blocked the formation of tumorspheres derived from SUM159 cells (Supplementary Figure 2b).

Non-canonical NF-κB activity is required for breast cancer cells to self-renew *in vitro*

As shown above, IKKα contributes to the self-renewal of basal-like breast cancer cells. IKKα typically acts in non-canonical NF-κB signaling (reviewed in Ghosh and Karin[15] and Hayden and Ghosh[16]), although it has recently been reported to promote canonical NF-κB signaling in breast cancer cells of the HER2+ subtype.[33] Knockdown with p100/p52 small interfering RNA (siRNA) efficiently reduced the precursor 100 kDa component and almost completely eliminated the processed p52 subunit (Figure 3a). Consistent with the IKKα knockdown results (Figures 2c and d), a statistically significant reduction in tumorsphere

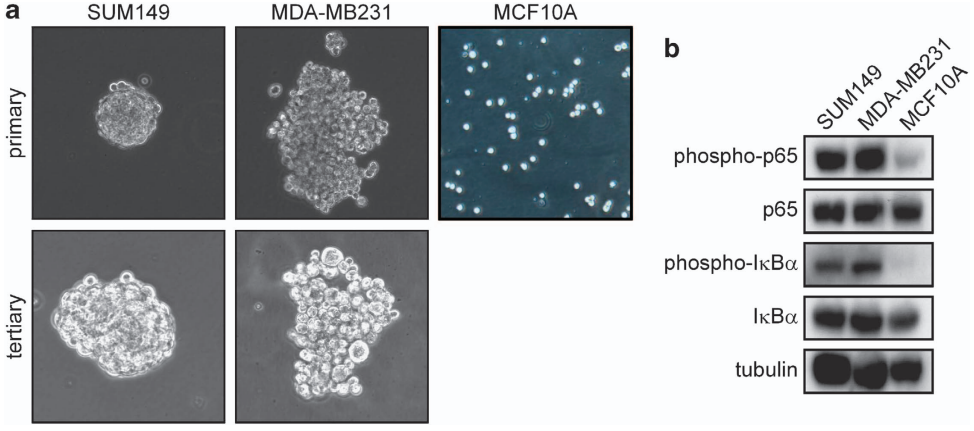

Figure 1. NF-κB signaling is preferentially activated in tumorsphere-forming breast cancer cells. (**a**) Primary and tertiary tumorspheres formed by the indicated bulk populations of basal-like and claudin-low breast cancer cells in serum-free culture on low-adhesion plates. (**b**) Phosphorylation of p65 and IκBα as markers of NF-κB activation in the indicated bulk populations of breast cancer cells (SUM149 and MDA-MB231) or immortalized breast (MCF10A) cells.

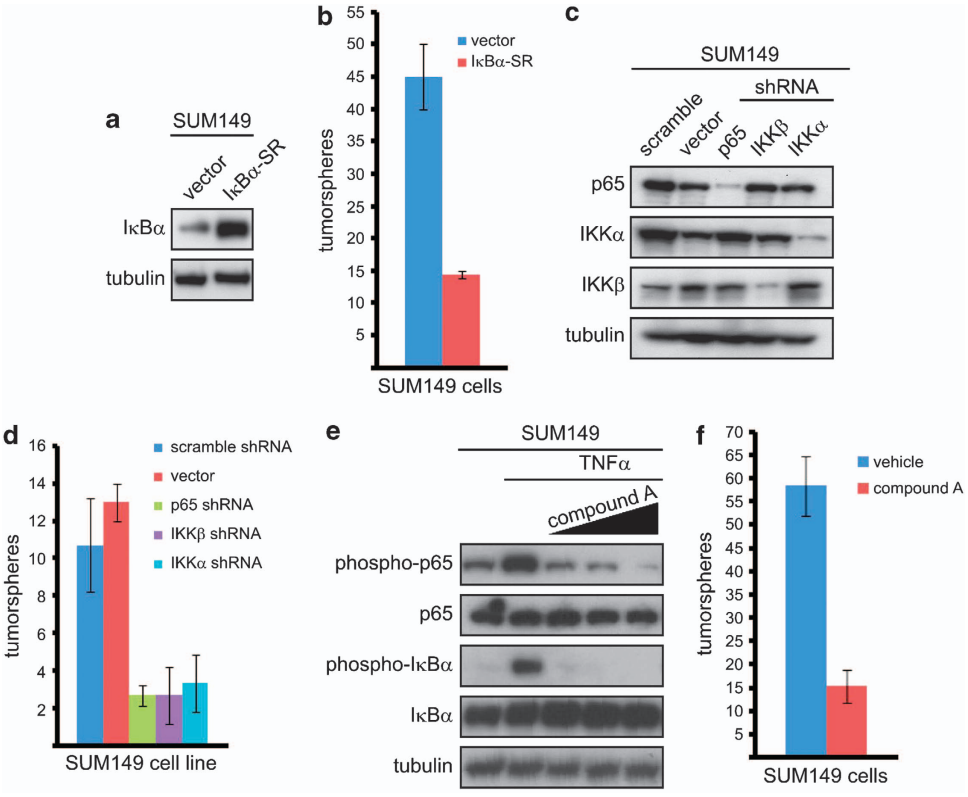

Figure 2. Canonical NF-κB signaling is required for basal-like breast cancer cells to efficiently self-renew. (**a**) Immunoblot of the indicated proteins in SUM149 cells stably expressing an empty vector or IκBα-SR. (**b**) Quantification of tumorspheres formed by 100 SUM149 cells expressing empty vector or IκBα-SR. (**c**) Immunoblot of the indicated proteins by 100 SUM149 cells stably infected with the indicated shRNA constructs. (**d**) Quantification of tumorspheres formed by 100 SUM149 cells stably expressing the indicated shRNA constructs. (**e**) Phosphorylation of p65 and IκBα as markers of activation of canonical NF-κB signaling in SUM149 cells pre-treated with increasing doses of compound A and then treated with tumor-necrosis factor-α. (**f**) Quantification of tumorspheres formed by 100 SUM149 cells treated daily with 5 μM compound A.

formation was observed in cells in which p100/p52 was inhibited by siRNA (Figure 3b). To further address a role for non-canonical NF-κB in promoting breast cancer cell tumorsphere formation, RelB was knocked down with siRNA (Figure 3a). As shown in Figure 3b, knockdown of this component of the non-canonical NF-κB pathway suppressed tumorsphere formation in both SUM149 and MDA-MB231 cells. These data, along with those shown in Figure 2, demonstrate that both canonical and non-canonical NF-κB are two signaling pathways that promote the self-renewal of breast cancer cells.

NF-κB promotes the self-renewal of breast cancer cells *in vivo*

In vivo, self-renewal is assayed by measuring the ability of cells to establish primary tumors when injected at limiting dilutions, which is specifically associated with the function of TICs.[2,34,35] To directly test the role of NF-κB in the self-renewal of basal-like breast cancer cells *in vivo*, SUM149 cells expressing empty vector or IκBα-SR at low (10^2 cells/100 μl) or high (10^6 cells/100 μl) density were prepared. These cells were injected into the mammary fat pad of nude mice and monitored as described in the methods. In these experiments, cells in which NF-κB signaling was deficient exhibited both delayed tumor onset and reduced overall tumor size (Table 1). Specifically, while the high density of SUM149 cells expressing empty vector formed palpable tumors at 6 weeks and reached an average tumor volume of 138 mm^3 by 10 weeks, the high density of SUM149 cells expressing IκBα-SR did not form palpable tumors until week 8 and these tumors maintained a significantly smaller size (11 mm^3) at week 10 (Table 1). Importantly, the low density of SUM149 cells expressing empty vector formed palpable tumors at week 9 and these tumors continued to increase in size at a significant rate (Table 1). Conversely, the low density of SUM149 cells expressing IκBα-SR did not form tumors (Table 1). These data demonstrate that NF-κB is required for xenograft-generated tumorigenesis in a context (limiting dilutions of cells, reviewed in Charafee-Jauffret *et al.*[2]) that depends significantly on self-renewal.

Canonical and non-canonical NF-κB are activated in breast cancer TICs and required for maintenance of TICs in the bulk population

The cell surface profile most commonly associated with breast cancer TICs is CD44+CD24−,[7,13,34,36–38] although CD44+, EpCAM+ or ALDEFLUOR-positivity (which depends on the activity of the enzyme ALDH1) are also indicative of this subpopulation of cells.[12,26,34,39–43] After examining the various cell surface profiles previously reported to enrich for TICs (data not shown), we chose to analyze CD44+ cells, which could be identified, isolated and, more importantly, self-renewed efficiently (see below). By calculating the percentage yield from the isolation protocol, bulk populations of both the SUM149 (Figures 4g and h) and MDA-MB231 cell lines (data not shown) were found to contain ~10% CD44+ cells. This percentage is lower than earlier reports,[2,12,42] although different groups have reported a range of the proportion of TICs even within the same cell line, particularly when different cell surface markers are utilized.[12,42]

Although the bulk population and CD44− cells each contain only a small proportion of cells that are positive for CD44, nearly all the cells in the CD44-isolated population robustly express CD44 (Figure 4a). This confirms that the isolation protocol successfully

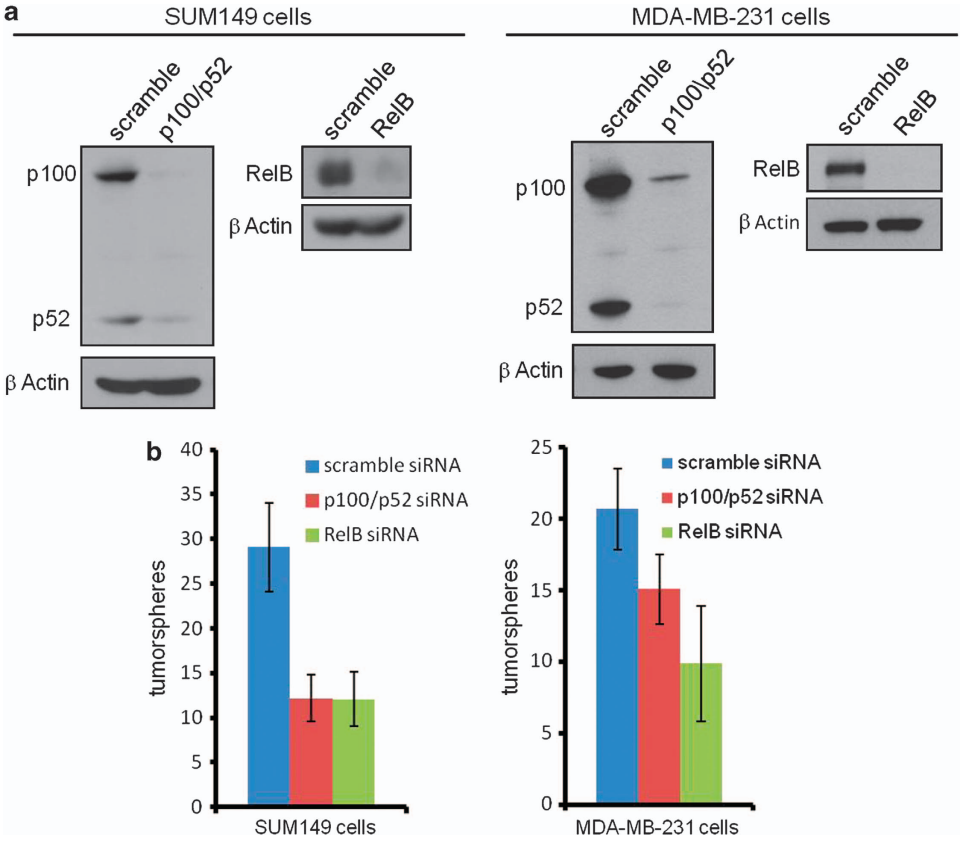

Figure 3. Non-canonical NF-κB signaling is required for basal-like breast cancer cells to self-renew. (**a**) Immunoblot of the indicated proteins in SUM149 and MDA-MB231 cells expressing scrambled siRNA or siRNA-targeting p100/p52 or RelB using the indicated antibodies. (**b**) Quantification of tumorspheres formed by 100 SUM149 or MDA-MB231 cells expressing the indicated siRNA constructs. *P*-values for the right panel of (**b**) are: $P = 0.0413$ for scramble siRNA compared with p100/p52 siRNA and $P = 0.0011$ for scramble siRNA compared with RelB siRNA.

Table 1. Inhibition of NF-κB signaling blunts tumorigenesis of limiting dilutions of basal-like breast cancer cells

	Average tumor volume (mm^3)				
	Week 6	*Week 7*	*Week 8*	*Week 9*	*Week 10*
SUM149 vector					
10^2 cells	—	—	—	1.33	8.25
10^6 cells	8.33	4.67	24.67	83.25	137.58
SUM149 IκBα*-SR*					
10^2 cells	—	—	—	—	—
10^6 cells	—	—	10.67	10.67	10.67

Quantification of the average tumor volume of xenograft tumors formed in the mammary fat pad of nude mice following injection of limiting dilutions of SUM149 cells expressing an empty vector or IκBα-SR.

enriches for CD44+ cells. Notably, CD44+ cells form tumorspheres significantly more efficiently than CD44− cells (Figure 4b), which demonstrates that the CD44+ isolation protocol enriches for TIC function. Given that NF-κB is required for the self-renewal of TICs (Figures 2 and 3), the activation of NF-κB was assessed in lysates from breast cancer TICs (CD44+ cells) compared with lysates isolated from the bulk population of cells and non-TICs (CD44− cells). Importantly, phosphorylation of p65 (Figure 4c) and levels of p52 (Figure 4d) are detected in CD44+ cells in both SUM149 and MDA-MB231 cells, indicating that both canonical and non-canonical NF-κB signaling is activated in breast cancer TICs. As before, the role of NF-κB in TICs of the claudin-low SUM159 cells was also assessed. Specifically, CD44+ cells isolated from SUM159 cells also exhibit elevated NF-κB activity, as measured by phosphorylation of p65 (Supplementary Figure 2c).

To determine if pathways upstream of NF-κB are also activated in breast cancer TICs, phosphorylation of IKKα and IKKβ was assessed by immunoblot of cellular lysates prepared from the bulk population of SUM149 cells and its TIC and non-TIC counterparts. In support of the concept that NF-κB activation is IKK-dependent in these cells, an increased signal for phosphorylated IKKα/β was observed in TICs isolated from SUM149 cells (Figure 4e). This fact is also consistent with evidence that canonical and non-canonical NF-κB pathways are important in TICs (Figures 2, 3, 4c and d). Finally, the activation of TAK1, a kinase that is an upstream activator of IKK,[44] was examined. Similar to IKK, phosphorylation of TAK1, which is indicative of its activation,[45] was enriched in CD44+ cells (Figure 4f).

Given that NF-κB is preferentially activated in breast cancer TICs (Figures 4a–f) and required for their self-renewal *in vitro* and *in vivo* (Figures 2 and 3 and Table 1), it was determined whether NF-κB is important in the maintenance of TICs in the bulk population of basal-like breast cancer cells. To this end, the percentage of TICs in the bulk population of SUM149 cells was determined in the presence or absence of overexpression of IκBα-SR. The resulting data showed that inhibition of NF-κB reduced the percentage of CD44+ cells by ~50% (Figure 4g). Similarly, stable knockdown of p65, IKKβ or IKKα each reduced the percentage of CD44+ cells by ~50% (Figure 4h). Taken together, these data demonstrate that both canonical and non-canonical

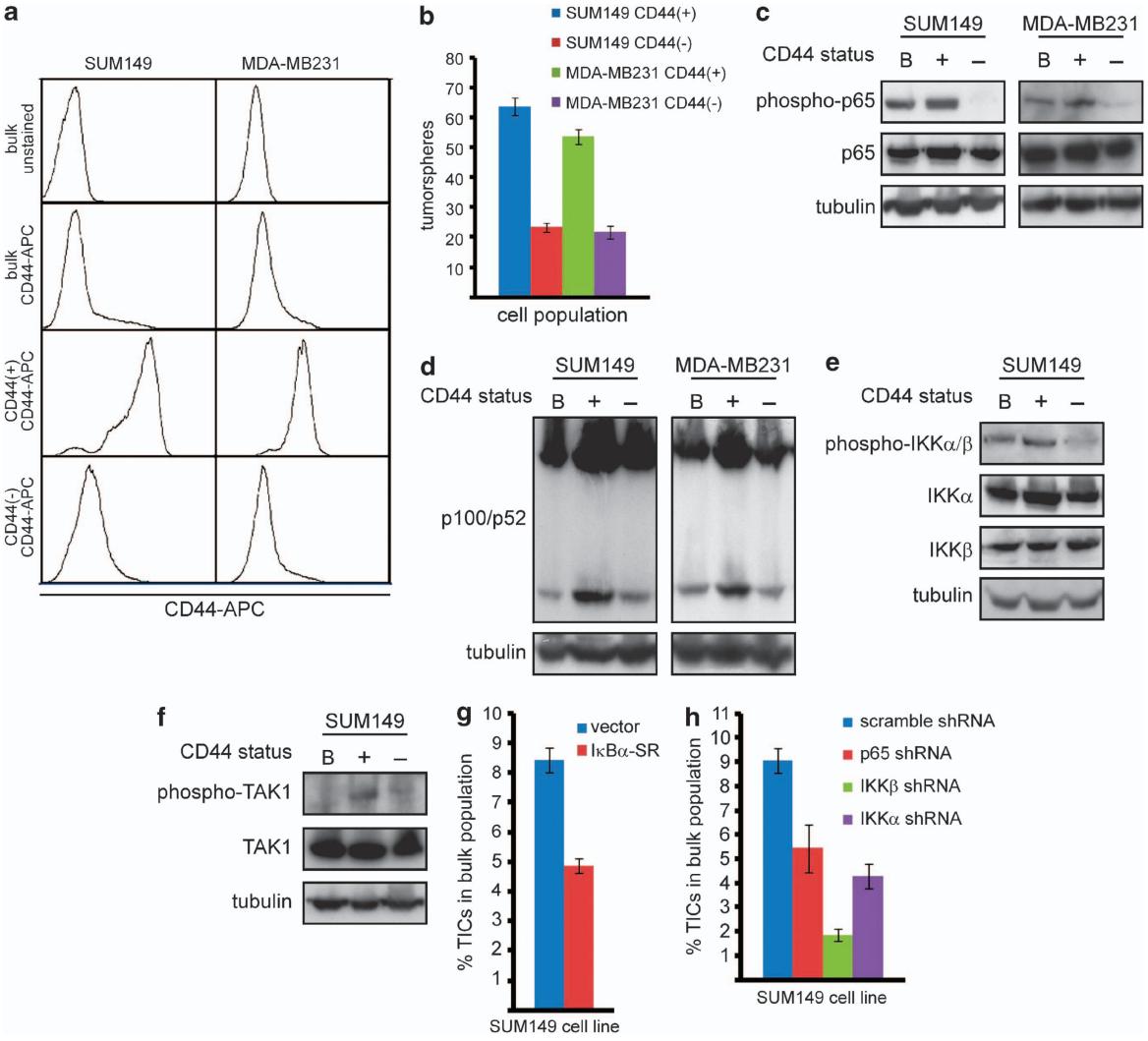

Figure 4. Canonical and non-canonical NF-κB signaling is preferentially activated in TICs and required for the maintenance of TICs in breast cancer cells. (**a**) Fluorescence-activated cell sorting analysis of the indicated populations of SUM149 cells stained with CD44-APC. (**b**) Quantification of tumorspheres formed by 100 cells from the indicated cell populations of SUM149 or MDA-MB231 cells. (**c**) Phosphorylation of p65 as a marker of activation of canonical NF-κB signaling in the indicated populations of SUM149 and MDA-MB231 cells. (**d**) Cleavage of p100 to p52 as a marker of activation of non-canonical NF-κB signaling in the indicated populations of SUM149 and MDA-MB231 cells. (**e**) Phosphorylation of IKKα and IKKβ in the indicated populations of SUM149 cells. (**f**) Phosphorylation of TAK1 in the indicated populations of SUM149 cells. (**g**, **h**) Percentage of TICs isolated from SUM149 cells stably expressing IκBα-SR (**g**) or the indicated shRNA constructs (**h**).

NF-κB signaling is preferentially activated in breast cancer TICs, consistent with observations from TICs in the bulk population of cells (Figures 2 and 3), and that NF-κB is important for the maintenance of the TIC population.

NF-κB promotes expression of markers of EMT in TICs and transforming growth factor-β-induced self-renewal

EMT is a process by which an epithelial cell releases from the basement membrane and transforms into a spindle-like, mesenchymal cell expressing vimentin and fibronectin (reviewed in Kalluri and Weinberg[46]). EMT has been demonstrated to promote the self-renewal of immortalized breast cells,[36,37] but this has not been examined in specific subtypes of breast cancer cells. NF-κB is one of the multiple signaling pathways implicated in the regulation of EMT, as activation of NF-κB is required for EMT that occurs during Ras-driven transformation.[47] Thus, we hypothesized that NF-κB promotes the function of breast cancer TICs by stimulating EMT. Expression of mesenchymal markers was analyzed in the bulk population of SUM149 or MDA-MB231 cells, and in CD44+ and CD44− cells. Both vimentin and fibronectin were detected in the bulk population as well as CD44+ and CD44− cells (data not shown). Importantly, expression of mesenchymal markers depends on NF-κB, as vimentin expression is decreased in the bulk population of SUM149 and MDA-MB231 cells expressing IκBα-SR (Figure 5a). To extend these results, knockdown of the RelA/p65 NF-κB subunit reduced vimentin and fibronectin expression in SUM149 cells (Supplementary Figure 3).

Transforming growth factor-β (TGFβ) is a well-characterized inducer of EMT and has been shown to promote the self-renewal of at least immortalized breast cancer cells.[36] To first confirm that TGFβ promotes self-renewal of basal-like breast cancer cells, 100 SUM149 cells expressing empty vector were plated on low-adhesion plates in serum-free media and treated every other day for 7 days with 10 ng/ml TGFβ or vehicle control. Such treatment induced a statistically significant, threefold increase in the number of tumorspheres formed by these cells (Figure 5b), suggesting that EMT is important for the function of TICs in basal-like breast cancer

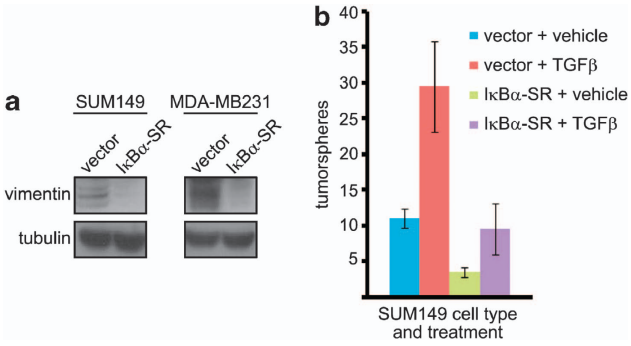

Figure 5. NF-κB activation promotes expression of markers of EMT in TICs and TGFβ-induced self-renewal of basal-like breast cancer cells. (**a**) Immunoblot of vimentin in SUM149 or MDA-MB231 cells stably expressing an empty vector or IκBα-SR. (**b**) Quantification of tumorspheres formed by 100 SUM149 cells stably expressing empty vector or IκBα-SR, followed by treatment with vehicle control or TGFβ.

cells. Importantly, it was investigated whether the ability of TGFβ-induced EMT to stimulate self-renewal of basal-like breast cancer cells depends on NF-κB. Specifically, SUM149 cells expressing IκBα-SR to inhibit NF-κB were also included in the above TGFβ experiment. As previously observed (Figure 2b), expression of IκBα-SR reduced the self-renewal of SUM149 cells (Figure 5b). TGFβ treatment of SUM149 cells expressing IκBα-SR resulted in the formation of a significantly smaller number of tumorspheres than TGFβ treatment of cells with proficient NF-κB signaling (Figure 5b). These data suggest that NF-κB promotes the self-renewal of basal-like breast cancer cells at least in part by stimulating EMT.

IL-1β and IL-6 stimulate the self-renewal of basal-like breast cancer cells downstream of NF-κB

Inflammatory cytokines that are NF-κB target genes have been demonstrated to be involved in self-renewal,[29,48,49] but the role of NF-κB in this process has not been thoroughly examined. We hypothesized that a subset of cytokine NF-κB target genes may be important to promote breast cancer TICs. To test this hypothesis, SUM149 cells were examined for expression of three inflammatory cytokines (IL-1β, IL-6 and IL-8), and for whether NF-κB is involved in regulating their expression in these cells. Analysis of mRNA expression by real-time PCR demonstrated that the IκBα-SR-expressing cells exhibit reduced levels of IL-1β, IL-6 and IL-8 mRNAs compared with vector control cells (Figure 6a). In addition, enzyme-linked immunosorbent assay (ELISA) analysis revealed significantly decreased levels of secreted IL-1β, IL-6 and IL-8 in the media of SUM149 cells, in which NF-κB is inhibited compared with the vector control cells (Figure 6b). These data confirm that NF-κB is critical for the expression and secretion of IL-1β, IL-6 and IL-8 in basal-like breast cancer cells.

To determine whether IL-1β, IL-6 or IL-8 promote the self-renewal of basal-like breast cancer cells, 100 SUM149 cells expressing an empty vector were plated on low-adhesion plates in serum-free media and treated every other day for seven days with either IL-1β, IL-6, IL-8 or vehicle control. Tumorspheres were enumerated following the completion of this treatment schedule. Importantly, treatment of SUM149 cells expressing empty vector with either IL-1β or IL-6 potently increased the number of tumorspheres by fourfold and twofold, respectively (Figure 6c). These data suggest that IL-1β and IL-6 are important modulators of the ability of basal-like breast cancer cells to self-renew. Treatment of SUM149 cells with CXCL7, the product of an NF-κB target gene, produced small increases in the ability of these cells to form tumorspheres (data not shown). Conversely, treatment with exogenous IL-8 failed to promote the formation of tumorspheres in control SUM149 cells (Figure 6c). Consistent with the results described above, addition of an IL-6 receptor antagonistic antibody suppressed tumorsphere formation ~25% and addition of recombinant IL-1β receptor antagonist suppressed tumorsphere formation ~30% (Supplementary Figure 4).

As IL-1β and IL-6 are known NF-κB target genes[16] and were upregulated by NF-κB in SUM149 cells (Figures 6a and b), we investigated whether NF-κB promotes self-renewal by inducing their expression and secretion. Specifically, we tested whether treatment with IL-1β or IL-6 can rescue the ability to self-renew in SUM149 cells in which NF-κB is inhibited. To do so, the above experiment was also performed using SUM149 cells expressing IκBα-SR. As before (Figure 2b), expression of IκBα-SR reduces the ability of untreated SUM149 to form tumorspheres (Figure 6c). Notably, treatment with either IL-1β or IL-6, but not IL-8, partially rescued the ability of IκBα-SR-expressing cells to form tumorspheres (Figure 6c). These data indicate that IL-1β and IL-6 promote self-renewal of basal-like breast cancer cells downstream of NF-κB.

DISCUSSION

Most solid tumors, including those of the breast,[50] are characterized by a hierarchy of cells including a sub-population of cells that can self-renew and give rise to the differentiated cells that comprise the bulk of the tumor. These TICs promote tumor initiation, cellular motility and invasiveness, tumor recurrence, and are typically radio- and chemoresistant. As such, characterizing the functional and phenotypic differences between the bulk population of cancer cells and TICs is critical in understanding tumorigenesis and gaining insight into new approaches for cancer therapy. The transcription factor NF-κB is widely implicated in a variety of oncogenic mechanisms in both hematological malignancies as well as solid tumors, including cancer cell proliferation, survival and metastasis.[18,19] Here, we have shown the involvement of NF-κB in promoting TICs in a basal-like breast cancer cell line, SUM149, and two claudin-low lines, MDA-MB231 and SUM159. Interestingly, both canonical and non-canonical NF-κB appear to be important in promoting TICs in these cancer cell lines. It will be interesting to determine if distinct or overlapping functions of these two pathways are operative in the maintenance of TICs. Work by others has indicated the association of NF-κB activity with other TICs. For example, prostate cancer TICs exhibit increased canonical NF-κB activity.[51] Also, published work indicates that canonical NF-κB signaling is important in TICs in the HER2+ breast tumor subtype.[25,26,52] One study utilized inhibitors that are not specific to NF-κB to suggest an involvement of NF-κB in promoting MCF7 breast cancer tumorspheres.[53]

We propose that NF-κB promotes the function of TICs through several mechanisms. First, NF-κB may promote TIC self-renewal by stimulating EMT. Second, NF-κB promotes TICs by stimulating the expression of cytokines, such as IL-1β and IL-6. Notably, IL-6 has been implicated in the induction of EMT in breast cancer cells.[54] Several reports support the finding that IL-6 promotes the function of TICs downstream of NF-κB. MCF10A cells transformed by Src gain the ability to form tumorspheres in a manner that depends on expression of IL-6.[29] In addition, it was found that treatment of a claudin-low breast cancer cell line with IL-6 increased the proportion of TICs.[49] That NF-κB upregulates the expression of many cytokines, and correspondingly can be activated downstream of these cytokines, indicates an important mechanism for sustaining NF-κB activation and promoting its TIC self-renewal properties in certain cancers. Additional roles for NF-κB in TICs likely include the upregulation of other key genes. Given the potential benefit of targeting TICs in breast cancer patients, the identification of NF-κB as a key regulator of TICs in

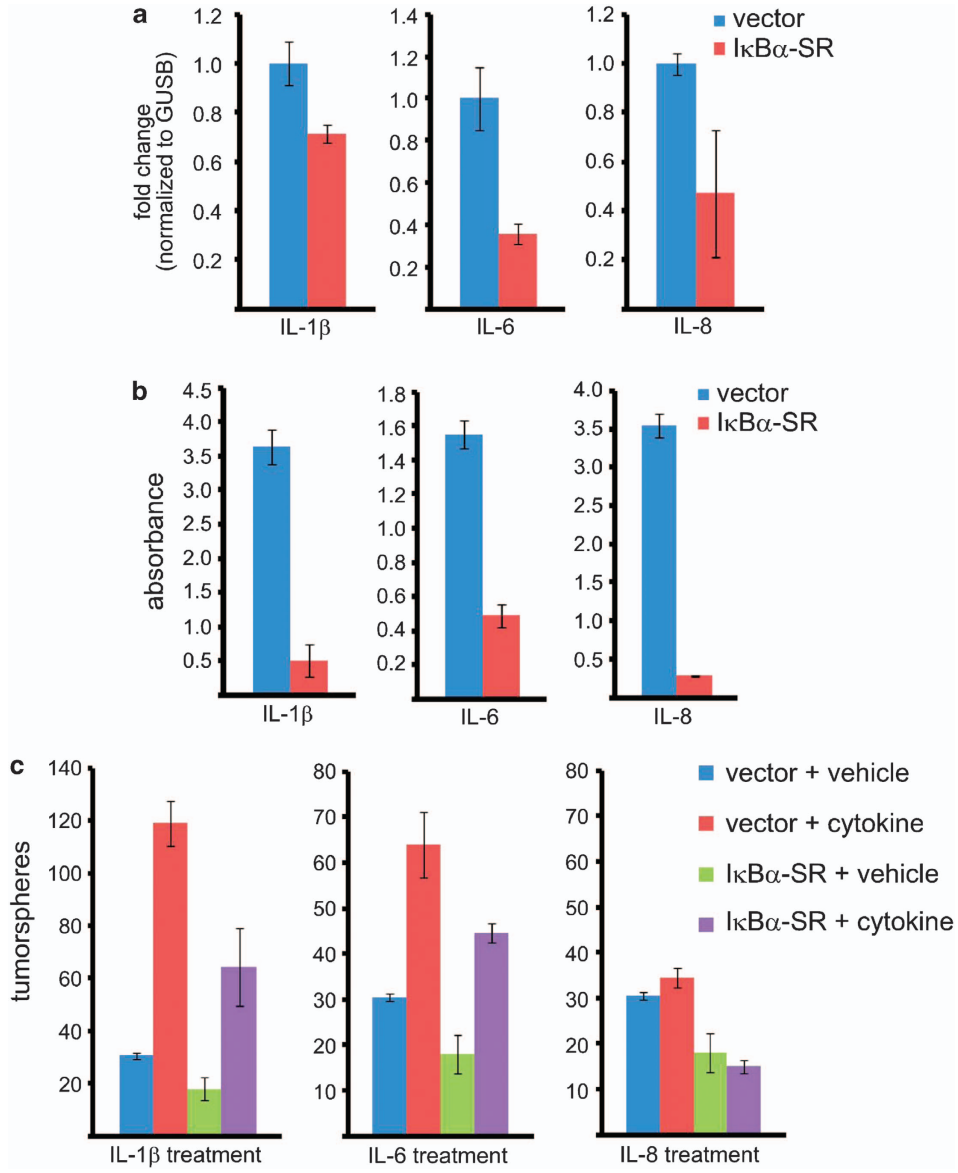

Figure 6. IL-1β and IL-6 stimulate the self-renewal of basal-like breast cancer cells downstream of NF-κB. (**a**) Real-time PCR showing expression of IL-1β, IL-6 or IL-8 in the bulk population of SUM149 cells stably expressing an empty vector or IκBα-SR. (**b**) ELISA analysis showing the abundance of IL-1β, IL-6 or IL-8 in the media of SUM149 cells stably expressing empty vector or IκBα-SR. (**c**) Quantification of tumorspheres formed by 100 SUM149 cells expressing empty vector or IκBα-SR, followed by treatment with IL-1β, IL-6, IL-8 or vehicle control.

basal-like and claudin-low breast cancer cells, along with previous work studying NF-κB in Her2+ TICs, represents a significant opportunity for the development of more effective chemotherapeutics for breast cancer.

MATERIALS AND METHODS

Cell culture and reagents

SUM149, MDA-MB231, SUM159 and MCF10A cells were maintained as described (Supplementary Materials). Details regarding shRNA constructs and antibodies are also found in Supplementary Materials.

Tumorsphere formation assay

Cells growing adherently in serum-containing media were trypsinized with TrypLE Select (Invitrogen, Grand Island, NY, USA) to generate a single cell solution and then enumerated using a hemocytometer. Subsequently, 100 cells per well were plated in 3 ml of Mammocult media (Stem Cell Technologies, Vancouver, BC, Canada) on six-well low-adhesion plates (Corning, Corning, NY, USA). Cells were treated with compound A, TGFβ, cytokines or vehicle controls as described in the text. The number of tumorspheres formed per well were counted visually. Cell density is a critical parameter in the tumorsphere formation assay and cells may aggregate if cell density is too high.[28] As such, we ensured that a disperse, low-density (100 cells in 3 ml of media) solution of cells was prepared for each tumorsphere formation assay to avoid cell aggregation. Furthermore, plates were not moved during the growth period to avoid cell aggregation. These precautions were taken to ensure the clonality of tumorspheres formed during this assay.[28]

Immunoblotting

See Supplementary Materials.

Fluorescence-activated cell sorting

Cell sorting was performed using a Beckman-Coulter (Dako, Carpinteria, CA, USA) CyAn and FlowJo software (TreeStar Inc., Ashland, OR, USA). See Supplementary Materials.

Isolation of TICs

Cells were trypsinized using TrypLE Select (Invitrogen) and dissociated by incubation in Accutase (Invitrogen) for 15 min at 37 °C. The resulting cell solution was passed through a pre-separation filter (Miltenyi Biotec, Auburn, CA, USA) to generate a single cell suspension. Cells were then incubated with 100 μl of Dead Cell Removal microbeads (Miltenyi Biotec) per 10^7 cells for 15 min at room temperature. Subsequently, the cell and microbead solution was resuspended in 20 ml of MACS buffer (Miltenyi Biotec) and passed through an LS column (Miltenyi Biotec) that was pre-moistened with MACS buffer and placed in a magnetized field. Live cells from the eluate were collected. Next, the resulting live cells were resuspended in 100 μl of MACS buffer per 10^7 cells and incubated with 75 μl of CD44 microbeads (Miltenyi Biotec) and 75 μl of FcR blocking reagent (Miltenyi Biotec) per 10^7 cells for 30 min at 4 °C. Subsequently, the cell and microbead solution was resuspended in 20 ml of MACS buffer (Miltenyi Biotec) passed through a LS column (Miltenyi Biotec) that was pre-moistened with MACS buffer and placed in a magnetic field. The CD44 − cells in the eluate were collected, the LS column was removed from the magnetized field and the CD44 + cells were collected from the column in 5 ml of MACS buffer.

MTT

The MTT assays were performed as previously described[55] using CellTiter cell viability reagent (Promega, Madison, WI, USA). See Supplementary Materials.

Enzyme-linked immunosorbent assay

ELISA kits (BD Biosciences, San Jose, CA, USA) were utilized according to the manufacturer's instructions.

Quantitative real-time PCR

Real-time PCR was performed and analyzed as previously described[56] using Taqman Gene Expression Assay primer-probe sets from Applied Biosystems (Grand Island, NY, USA).

Xenograft tumor formation

Cells were trypsinized with TrypLE Select (Invitrogen), a single cell solution was generated and the cells were enumerated using a hemocytometer. Cell solutions were generated in 50:50 media:Matrigel (BD Biosciences) at concentrations of 10^6 or 10^2 cells/100 μl. Further, 100 μl of the resulting solutions were orthotopically injected into the mammary fat pad of athymic nude-Foxn1^{nu} mice (Harlan Laboratories, Indianapolis, IN, USA). Injection of each cell solution was repeated in triplicate. All murine studies were conducted in accordance with guidelines from the UNC Institutional Animal Care and Use Committee on approved protocol 08–266.

ABBREVIATIONS

EMT, epithelial-to-mesenchymal transition; IκB, inhibitor of κB; IKK, IκB kinase; IL, interleukin; NF-κB, nuclear factor-κB; TIC, tumor-initiating cell; TGFβ, transforming growth factor-β

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We would like to thank members of the Baldwin lab for helpful discussions and comments. MFK and JWB were supported by a T32 National Research Service Award (5-T32-CA009156-37) awarded to the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. MFK was also supported by an F32 National Research Service Award (1F32CA153439-01) from the National Institutes of Health and a post-doctoral Fellowship from the American Cancer Society (120296-PF-11-093-01-DDC). JWB was also supported by an F32 National Research Service Award (1F32CA162628-01) from the National Institutes of Health. Research support was provided by National Cancer Institute, grants CA138937 and CA73756, and by Department of Defense grant BC073048. Additional research support was provided by the Samuel Waxman Cancer Research Foundation.

Author contributions: Dr MFK performed experiments and wrote the manuscript. Dr JWB performed key experiments and edited the manuscript. Dr CLL performed key experiments. KSC performed the tumor xenograft studies. Dr ASB edited the manuscript.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

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BC074027-3035

NF-kappaB MAY PROMOTE THE TUMOR-INITIATING CELL PHENOTYPE IN BREAST CANCER

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A breast tumor is composed of a heterogeneous population of cells, including a subset of cells termed tumor-initiating cells (TICs). These cells are particularly enriched in the basal and claudin-low subtypes of human breast cancer. TICs are characterized by robust self-renewal, elevated motility and invasiveness in vitro that translate to high metastatic potential in vivo, and radio- and chemoresistance. Thus, they may underlie a significant portion of the lethality of cancer and represent an important target for clinical intervention. Thus, it is important to understand signaling mechanisms that drive breast cancer-initiating cells.

The NF-kappaB (NF-kB) family of transcription factors is known to be involved in breast cancer. For example, we have shown that Her2, the EGFR family member associated with approximately 25% of breast cancer, is a potent activator of NF-kB to promote cancer-cell invasion. Others have shown that cells derived from murine mammary tumors in which NF-kB has been inactivated exhibit reduced self-renewal, and NF-kB is known to promote invasion and chemoresistance at least in some contexts in vitro. Mechanistically, NF-kB can mediate the epithelial-to-mesenchymal transition, the only cellular process yet known to promote the conversion of cells to TICs. We hypothesize that a distinct subset of NF-kB target genes promotes the TIC phenotype.

Our data support the hypothesis that NF-kB plays an important role in breast cancer TICs. Specifically, we have successfully isolated TICs (CD44+CD24-cells) from human breast cancer cell lines. Importantly, these cells more robustly express phosphorylated p65, a marker of NF-kB activity, than the bulk population. We also have successfully formed mammospheres from breast cancer cell lines in cell culture and observed that genetic inhibition of several components of NF-kB signaling results in reduced mammosphere formation. Finally, such inhibition of NF-kB results in a significant reduction of the percentage of TICs in the bulk population. Additional ongoing experimentation, both in vitro and in vivo, will address the contribution of the NF-kB pathway to the maintenance of breast cancer TICs.

In summary, our results suggest that NF-kB is integral to the TIC phenotype. Given the role TICs are thought to play in the initiation and progression of breast cancer, inhibition of NF-kB may be a beneficial therapeutic strategy and potentially improve clinical outcomes.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0378.